Novel Regulators of Feeding and Cardiovascular Physiology in Fish

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ABSTRACT

Nesfatin-1, an 82 amino acid anorexigen is encoded in a secreted precursor, nucleobindin-2 (NUCB2). NUCB2 was named so due to its high sequence similarity with nucleobindin-1 (NUCB1). It was recently reported that NUCB1 encodes an insulinotropic nesfatin-1-like peptide (NLP) in mice. Irisin, a muscle protein is encoded in its precursor fibronectin type III domain containing 5 (FNDC5) and released into blood from skeletal muscle. Here we aimed to characterize NLP and irisin in fish, and to study whether these are novel regulators of feeding and cardiovascular functions in zebrafish and goldfish. Western blot analysis and immunohistochemical studies determined the expression of NUCB1/NLP in central and peripheral tissues of goldfish. Administration of rat and goldfish NLP at 10 and 100 ng/g body weight doses caused potent inhibition of food intake in goldfish. NLP also downregulated the expression of preproghrelin and orexin-A mRNA, and upregulated cocaine and amphetamine regulated transcript (CART) mRNA in goldfish brain. Intraperitoneal (I.P) administration of NLP reduced cardiac functions in zebrafish and goldfish, downregulated irisin, and RyR1b mRNA expression in zebrafish. Irisin was detected in zebrafish heart and skeletal muscle. Single I.P. injection of irisin did not affect feeding, but its knockdown using siRNA caused a significant reduction in food intake. Knockdown of irisin reduced ghrelin and orexin-A mRNA expression, and increased CART mRNA expression in zebrafish brain and gut. Meanwhile, injection of irisin (0.1 and 1 ng/g B.W) increased cardiac functions, while knockdown of irisin resulted in reverse effects on cardiovascular physiology. Administration of propranolol attenuated the effects of irisin on cardiac physiology. Collectively, my research discovered that NLP and irisin modulate food intake and cardiac physiology in fish. Future studies should focus on the mechanisms of action of NLP and irisin in regulating metabolism and cardiovascular biology in fish.

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DEDICATION

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LIST OF ABBREVIATIONS

A

ANS - Autonomous nervous system

ACh – Acetylcholine

ANP - Atrial-natriuretic peptide

ACTH - Adrenocorticotropic hormone

AV - Atrioventricular Node

ATP – Adenyl pyrophosphatase

AgRP – Agouti Related Protein

AMPK - Adenosine monophosphate-activated protein kinase

ANOVA – Analysis of Variance

AMPK - Adenosine monophosphate-activated protein kinase

B

BNP - Brain-natriuretic peptide

β-blockers - Beta-adrenergic blockers

B. W **–** Body Weight

BDNF – Brain – derived neurotrophic factor

C

CCK - Cholecystokinin

CART - Cocaine and Amphetamine- Regulated Transcript

CO - Cardiac output

COX-2 - cyclooxygenase

$Ca2^{+}$ - Calcium

CCh - carbachol

CRF - R1 - Corticotropin-releasing hormone receptor 1

CHD - Coronary heart disease

E

ERK - Extracellular Signal-Regulated Kinase

F

FNDC5 - Fibronectin type III domain-containing protein 5

FOXL2 - Forkhead box L2

G

GH - Growth Hormone

GnRH - Gonadotropin-releasing hormone

GnRH - Gonadotropin-releasing hormone

GIP - Gastric inhibitory polypeptide

GLP-1 - Glucagon like peptide-1

GHS – R - Growth hormone secretagogue receptor

GAL – Galanin

GPCR - G-protein-coupled receptors

GI - Gastrointestinal

H

HUFA - Highly unsaturated fatty acids

HPI - Hypothalamus-pituitary-interrenal

HPG - Hypothalamic-pituitary- gonadal

HPO - Hypothalamus-pituitary-ovarian

HR - Heart rate

I

ICV - Intracerebroventricular

IR - Immunoreactivity

I.P – Intraperitoneal

IGF - Insulin-like growth factor 1

M

MSH - Melanocyte-stimulating hormone

mAchRs - Muscarinic acetylcholine receptor

N

NMU - Neuromodin U

NPY- Neuropeptide Y

NLP – Nesfatin - 1 Like Peptide

NE – Norepinephrine

NUCB1 – Nucleobindin - 1

NUCB2 – Nucleobindin – 2

Nesfatin - 1 - NEFA/Nucleobindin - 2 encoded satiety and fat - influencing protein

NLT - Nucleus lateralis tuberis

Na - Sodium

O

O_2 – Oxygen

P

PUFA - Polyunsaturated fatty acids

PYY - Peptide YY

POMC - Proopiomelanocortin

PGC1-α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PC – Prohormone convertase

R

RyR – Ryanodine

RNA - Ribonucleic acid

RPD – Rostral pars distalis

S

SA node - Sinoatrial node

SV - Stroke Volume

SA - Sinoatrial Node

SERCA - Sarco/endoplasmic reticulum Ca^{2+}-ATPase

SEM – Standard error of mean

T

TSH - Thyroid-stimulating hormone

TMS - Tricaine methanesulfonate

V

VIP - Vasoactive intestinal peptide

U

UBM – Ultrasound biomicroscopy

Z

ZT – Zeitgeber Time

Chapter 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Hormones and naturally occurring proteins play a major role in regulating physiological processes in animals, especially in vertebrates that possess more complex neural and endocrine regulatory networks [1-5]. A multiple redundant milieu of endocrine factors is essential for energy homeostasis [1, 6]. Food intake, an important aspect of energy balance is under the control of a number of hormones [7-17]. Similarly, hormones modulate cardiovascular physiology [18-22]. Myocardial function is entirely dependent on the balance between the work of the heart necessary to meet the requirements of the body and energy required to synthesize to sustain excitation-contraction coupling [23]. The heart has high energy demands to maintain cellular processes such as ion transport and intracellular Ca^{2+} homeostasis [23]. During endurance, the heart has the ability to use more than 90% of its oxidative capacity establishing a strong relationship between oxygen consumption, energy homeostasis and cardiac work driven by ATP-dependent pathways [23-25]. Some hormones are key endogenous players in maintaining both energy balance and cardiovascular biology. Examples include ghrelin, growth hormone (GH), adrenocorticotropic hormone (ACTH), orexins, calcitriol, aldosterone and angiotensin [26-30]. In the post-genomic era, a large number of peptides were discovered and many of them were found to have roles in energy balance and cardiac physiology. One such naturally occurring orphan ligand (receptor unknown) is nesfatin-1 [31, 32].

In 2006, nesfatin-1 (NEFA/nucleobindin-2 encoded satiety and fat-influencing protein-1) an 82-amino acid peptide encoded in the N-terminal region of its precursor, nucleobindin-2 [32, 33] was discovered. NUCB2 is cleaved by prohormone convertases into three different peptides, namely nesfatin-1 (82 amino acids), nesfatin-2 (85-163 amino acids) and nesfatin-3 (166-396 amino acids), respectively. Among these peptides, only nesfatin-1 has been shown to have a biological activity. The 30-amino acid mid-segment of nesfatin-1 is considered as the bioactive core, which has shown to affect appetite, the hypothalamus-pituitary-ovarian axis, and to modulate intracellular Ca^{2+} signaling in mammals [34]. Upon its discovery, nucleobindin-2 was given its name due to its high sequence similarity to another secreted protein, nucleobindin-1 (NUCB1). NUCB1 has been localized in the stomach, testis, ovary, adrenal glands etc. [35]. Our lab, for the first time, proposed the possible presence of an endogenous nesfatin-1-like peptide (NLP) in mice, and reported its insulinotropic action on mice pancreatic beta cells. NUCB1 has been shown to have a vital role in maintaining $Ca2^{+}$ homeostasis and interact with G-proteins and cyclooxygenases [36-38]. **Chapters 2** and **3** of this thesis provide novel information on NLP in regulating feeding and cardiac physiology.

A second example of an orphan ligand known to be involved in the physiology of mammals is irisin, an exercise-induced myokine cleaved from its 212 amino acid precursor fibronectin type III domain containing 5 (FNDC5) [39-41]. FNDC5 is regulated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha), which forms an integral part of the muscle post-exercise and causes an increase in energy expenditure in mammals [39]. PGC-1 alpha is a transcriptional co-activator that plays an important role in regulating mitochondrial function and oxidative phosphorylation. PGC-1 alpha is a major regulator of adipocyte browning

and thermogenesis [42-45]. Processing of FNDC5 by PGC-1 alpha triggers the release of irisin into circulation [39, 46]. In zebrafish, irisin promoted angiogenesis and modulates matrix metalloproteinase activity through the extracellular signal–regulated kinase (ERK) signaling pathway [47]. In mammals, intra-hypothalamic injection of irisin (50-200 nmol/L) decreased feed intake and upregulated cocaine and amphetamine regulated transcript (CART) and proopiomelanocortin (POMC) mRNA expression [48]. Intraperitoneal injection of irisin suppressed feed intake and modulated appetite regulatory peptides in goldfish [49]. **Chapter 4** reports irisin regulation of feeding and feeding regulatory peptides in zebrafish. Meanwhile, **chapter 5** describes irisin effects on cardiac parameters and muscle proteins of interest in zebrafish. In an effort to further our understanding on how irisin modulates cardiac physiology, we studied the role of adrenergic signaling in modulating CV effects of irisin, and this work is detailed in **chapter 6** of this thesis.

This review is primarily focused on the regulation of food intake and cardiovascular physiology, two focus areas of my thesis research. Due to the extensive list of hormones that regulate both processes, only selected factors known in fish, and studied in my research are discussed in detail.

1.2. FEEDING AND METABOLISM IN FISH

Food intake and nutrient composition determine fish metabolism and growth [50, 51]. Metabolic hormones, and the growth hormone insulin-like growth factor (GH-IGF) axis regulate feeding, metabolism and growth [52, 53]. Nutrient availability and proper endocrine signalling help organisms maintain metabolic homeostasis. The following sections will elaborate on the importance of hormones and nutrient requirements in fish metabolism.

1.2.1. Hormones that Regulate Feeding in Fish

Feeding and metabolism in fish are tightly interconnected processes [17, 54, 55]. Nutritional factors gained through feeding, the metabolic processing of food, and the partitioning of energy substrates are essential to maintain reproduction. The feeding regulatory and metabolic hormones mainly act on the hypothalamus, the main metabolic control center of the brain [17]. The hypothalamus is the primary center that play an important role in modulating feed intake, by regulating nutrient sensing systems that modulate energy metabolism directly or indirectly through endocrine factors [17, 54, 55]. Other regions of the brain including ventral telencephalon and gustatory nucleus also play an important role in feeding [56], and olfactory tract lesions were found to reduce feeding in teleosts [56, 57]. Regulation of feeding and metabolism [17, 58] in fish occur through an intricate, redundant and multifactorial hormonal milieu. Hormones that stimulate feeding are named orexigens, while those that inhibit are anorexigens.

Metabolism plays an important role in energy homeostasis and appetite behavior in both mammals and non-mammals. The regulation of metabolism involves integration of endogenous and exogenous factors that provide adequate supply of energy to carryout biological processes.

The endocrine system produces hormones and coordinates the activity of organs. Hormones, primarily act on the brain, mainly hypothalamus to produce key factors that regulate feeding activity in vertebrates [17]. In fish, there are numerous peptide regulators of appetite regulation, including neuropeptide Y (NPY), ghrelin, orexin-A, proopiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART) and nesfatin-1.

Neuropeptide Y (NPY), a 36-amino acid orexigen, has been identified in a large number of fishes with strong expression identified in pituitary, pancreas, intestine and nerve fibers [59-63]. Intracerebroventricular injection (ICV) injection of NPY stimulated food intake in goldfish, salmon and catfish [64, 65]. In goldfish, administration of NPY stimulated food intake, while food deprivation caused an increase in NPY mRNA in the hypothalamus [66, 67]. Meanwhile, re-feeding after 72-h reversed the effects of food deprivation on brain NPY mRNA in goldfish and catfish [62, 67]. Exposure to high carbohydrate or fat diets exhibited changes in the expression of NPY in brain suggesting a strong relationship between NPY and diets in fish [68].

Ghrelin, a gut hormone has been identified in numerous fishes has been shown to stimulate food intake [28, 69, 70]. The physiological functions of ghrelin is exerted by binding to the growth hormone secretagogue receptor-1a (GHS-R1a/ghrelin receptor) [71]. Ghrelin mRNA has been widely expressed in fish gut and also moderate levels in the brain [72-74]. The cDNAs of ghrelin receptor has been identified in pufferfish and seabream and are highly expressed in the pituitary and brain [75, 76]. Central and peripheral injections of ghrelin resulted in the stimulation of food intake in goldfish [73, 77]. In goldfish, 7 days of food deprivation increased preproghrelin mRNA expression in the hypothalamus and gut. Increased levels of ghrelin

modulated liver glycogenolysis in burbot during spawning of burbot [78]. These results suggest a major role for ghrelin in feeding and energy metabolism in fish. Other physiological functions of ghrelin include GH release from the pituitary, glucose regulation and lipid metabolism, and cardiovascular effects in fish [79].

Orexins/hypocretins, are neuropeptides that contains two forms, orexin-A and orexin-B, both cleaved from the same precursor, preproorexin [29, 80]. In fishes, mRNAs encoding the precursor, preproorexin have been identified in pufferfish and zebrafish [81, 82]. In fish, orexins possess modulatory effects on various appetite regulators such as NPY, galanin (GAL) and CART [29, 83]. In fish, high doses of orexin A stimulated NPY expression and increased food intake indicating that NPY and orexin-A are dependent on each other in affecting feed intake and feeding behavior in goldfish [84]. Administration of CART and leptin inhibited orexin-A induced feeding, suggesting a functional interdependence between orexin and anorexigenic peptides in controlling energy balance in teleosts [11, 85]. Other physiological roles of orexins include regulation of wakefulness, hyperphagia, sleep cycles, daily rhythm, locomotor activity and energy homeostasis in goldfish and zebrafish [11, 82, 86].

POMC, which encodes melanocyte stimulating hormone (MSH) is a 267-amino acid neuropeptide secreted by the corticotropic cells of the anterior pituitary as well as by melanotropic cells of pars intermedia in teleosts [87]. In fish, POMC encodes for number of MSHs: α- MSH and β-MSH in bony and cartilaginous fish [88-92]. In goldfish, POMC neurons have been widely distributed within the lateral tuberal nucleus and central administration of MSH receptors suppressed feeding intake in 24 hour food deprived goldfish in a dose dependent

manner [89]. α-MSH suppresses food intake, modulates lipase activity and enhances energy expenditure in fish [87, 89, 93].

CART is an anorectic neuropeptide encoded in either a 117-amino acid pro-CART or a 120-amino acid pro-CART in goldfish [11]. In goldfish, CART mRNAs are widely expressed in brain, pituitary and other peripheral tissues such as eye, gonads and kidney [11, 84]. Food deprivation caused a decrease in CART mRNA levels in the hypothalamus, and CART II mRNA expression in the olfactory bulbs [94]. ICV injections of CART (62-76) decreased food intake in goldfish [11]. CART (55-102) injection decreased NPY and orexin-A mRNAs and suppressed feeding in fish [94].

Nesfatin-1, an 82-amino acid peptide encoded in nucleobindin-2 is an anorexigenic peptide shown to reduce feeding and regulation of hypothalamus-pituitary ovarian axis in mice [34]. Nesfatin-1 is processed from precursor nucleobindin-2 (NUCB2) and in fish two isoforms of NUCB2 have been identified (NUCB2A and NUCB2B) [95]. NUCB2A mRNA has been widely expressed in brain (hypothalamus), liver, gonads, liver and hepatopancreas of goldfish and Ya fish [95, 96]. In goldfish, nesfatin-1 was detected in the hypothalamus localized in the nucleus lateralis tuberis (NLT) by immunohistochemical studies [95]. Peripheral or central administration of nesfatin-1 suppressed feeding and affected appetite regulatory peptides such as orexin-A, and ghrelin suggesting an anorectic and metabolism role in goldfish [95]. Post feeding, NUCB2A mRNA expression was increased in the intestine and hypothalamus, while upon food deprivation or short term fasting, NUCB2A mRNA expression was found to be increased in the hepatopancreas of goldfish [95]. In zebrafish, nesfatin-1 is expressed abundantly in the liver and

anterior GI tract. NUCB2/nesfatin-1- like immunoreactivity is expressed in zebrafish gut and co-localized with ghrelin [97]. Food deprivation for a period of 7-days resulted in decreased NUCB2 mRNA in zebrafish gut, brain and liver, supporting evidences that nesfatin-1 possess anorexigenic roles in fishes [97]. In addition to the hormones that regulate feeding and energy metabolism, nutrient availability is required for the adequate supply of energy to maintain metabolic homeostasis.

1.2.2. Nutrient Requirements

Nutrients are classified as organic compounds that are essential for any living organism to support reproduction, growth and maintenance of life processes [98]. The availability of nutrients is critical to support cellular energy requirements [99]. Nutrients can be divided into two subclasses: macronutrients and micronutrients. Macronutrients are further classified into carbohydrates, lipids and proteins/amino acids, while micronutrients are composed of vitamins and minerals [100]. Nutritional requirements are species specific, and are determined by various external and internal factors including environmental conditions, growth, and reproductive status of the organism [101]. Due to the diet and feeding studies included in this thesis research, we will briefly discuss how macronutrients and micronutrients are essential for fish metabolism, growth and reproduction.

1.2.2.1. Macronutrients

Proteins are dietary macronutrients that are essential for growth, fish digestion and other life processes. All cell types contain proteins, and synthesize proteins throughout their life cycle. The requirement of protein is highest for young growing animals and gradually decreases as they mature due to the fact that adequate amount of proteins is only required to maintain body tissues. Proteins are digested or hydrolyzed and released as free amino acids which form an essential component of the diet of all animals to supply energy [102]. Amino acids are essential components of nutrients required by all animals to build new proteins responsible for growth and reproduction and replace existing proteins to maintain homeostasis. In addition to these, some proteins are enzymes that play a vital role in catalyzing various reactions, muscle metabolism and transport of nutrients and oxygen across membranes [99, 103].

In fish, carbohydrates stored as glycogen is vital for the organism to meet energy demands whenever necessary. The energy source in carbohydrates is glucose, a monosaccharide (6 carbon atoms) that is important for the regulation of metabolism in vertebrates. The availability of glucose required for cellular processes is met by ingestion of glucose by the organism, or by its breakdown from storage [104, 105]. In recent years, various studies in fish have been demonstrated the discovery of glucose sensing mechanisms in endocrine tissues including the hypothalamus, pancreatic endocrine cells and intestine [50]. In fish, the presence of glucokinase dependent glucosensors regulate feed intake, and modulate appetite regulatory peptides [104-108]. Brain areas that secrete AgRP/NPY and POMC neurons express glucokinase, suggesting an interlink between glucosensors and appetite regulatory neuropeptides in fish [109]. With respect to energy metabolism, central administration of glucose resulted in increased glucokinase activity in the Brockmann bodies (centre where pancreatic endocrine cells are located in most fish species) of rainbow trout [110]. Similarly, in zebrafish, exposure to high glucose stimulated the gene expression of insulin [111].

Lipids (fat) are energy rich fatty acid compounds that are used as a substitute for protein in most aquaculture feeds. In fish, lipids are considered as the main nutrient component that is responsible for regulating various physiological processes necessary for growth and reproduction [112]. In fish diet, lipids constitute around 10-20 % consisting of essential fatty acids, serve as energy providing nutrients necessary to allow protein to be efficiently utilized for fish growth and metabolism and avoiding excessive lipid deposition in fish tissues. Lipids include fatty acids and triglycerols. Fish requires omega-3 and omega 6 (n-3; n-6) fatty acids. Fatty acids can be grouped as saturated fatty acids, polyunsaturated fatty acids (PUFA), and highly unsaturated

fatty acids (HUFA). Several studies have shown the role of fatty acid in regulating feed intake in fish. In rainbow trout and grass carp, fatty acid sensors have been detected in the hypothalamus, the main metabolic centre of the brain that regulate feeding intake and metabolism in fish [113, 114]. In rainbow trout, fatty acid sensors have been reported to decrease lipogenic and fatty acid oxidation. In rainbow trout, use of lipolysis inhibitors led to the inhibition of fatty acid sensing systems, decreased fatty acid levels and inactivation of hypothalamus-pituitary-interrenal axis (HPI axis) [115].

1.2.2.2. Micronutrients

Vitamins are organic compounds that are important components of the fish diet for metabolism, growth and health. These organic compounds are not synthesized by the animal and must be supplied as part of the diet. The omission of a single vitamin from the diet of the species might result in vitamin deficiency, ultimately leading to mortality. Vitamin deficiencies lead to retarded growth, improper bone mineralization, scoliosis and reduced growth and appetite.

Minerals are classified as inorganic compounds in the diet that play a significant role in various body functions including bone formation, regulation of cellular metabolism and acid-base balance [116]. Minerals are grouped into macrominerals and microminerals based on the requirement in the diet and amount present in fish. Macrominerals include calcium, sodium, phosphorus, chlorine and magnesium that regulate extracellular osmotic balance, maintaining osmotic pressure and development of bone structure. Microminerals include iodine, zinc, iron and manganese play an important role in absorption and transport of nutrients in fish. Fishes can

absorb many minerals by the process of osmoregulation through their gills and skin, thereby avoiding mineral deficiencies through their diet [116].

1.3. CARDIOVASCULAR PHYSIOLOGY IN FISH

Heart is the first organ to develop and function during the embryonic development in vertebrates. The development of heart begins with the growth of myocardial and endocardial progenitor cells (atrial and ventricular) [117]. The atrial progenitor cells are localized ventrally when compared to the ventricular progenitor cells [118]. Developmental size of the myocardial progenitor cells is restricted by retinoic acid signaling pathway [119]. Heart pumps blood to different tissues across the body to provide adequate nutrients and supply of oxygen, and also to remove waste materials. The wall of the heart is subdivided into three layers, the epicardium, myocardium and pericardium. Human heart consists of atria (left and right), ventricle (left and right), aorta, vena cava (superior and inferior), pulmonary arteries and veins (left and right), mitral valve and tricuspid valve [120].

Fish heart exhibits slightly different anatomical structure when compared to mammalian heart, and is subdivided into four chambers: sinus venous, atrium, ventricle and bulbus arteriosus. The fish heart is also comprised of cardiac muscle and elastic muscle [121]. Ventricular layer of fish heart is single chambered constituting inner spongiosa layer and outer compacta layer. Atrial wall of heart is contractile and thin layered containing afferent and efferent arteries, where the former has a thick wall and facilitates transport of blood from heart to gill tissue while the latter transport blood from gills to different parts of the body [121].

Circulatory system in fish is closed with slight variation in its characteristics depending on the species. In this type of circulatory system, the deoxygenated blood enters into the right atrium (systemic circulation) from the sinus venosus [27]. The blood then enters the right

ventricle wherein it is pumped out (pulmonary circulation) through the pulmonary artery. The blood in the lungs receives adequate supply of oxygen and enters the left atrium through pulmonary veins [27]. The blood then enters into the left ventricle via mitral valve and into the bulbus arteriosus. From the bulbus arteriosus, the oxygenated blood passes through arteries and capillaries to reach various cells, while the deoxygenated blood enters into the right atrium to repeat the whole process. Ventral aorta transport deoxygenated blood from the aorta located ventrally onto the gills, while the dorsal aorta assists in transporting oxygenated blood to the aorta and circulated to the different parts of the body.

The electrical signaling of the heart is mediated by the sinoatrial node (SA node), atrioventricular node (AV node), and Purkinje fiber bundles. Atrial contraction is activated by the signals originating from the SA node. The signals are then conducted to the Purkinje fiber bundles that are present in the ventricular walls of the heart causing an action potential. This action potential results in the myocardial wall to undergo ventricular contraction. Systole is the period when heart undergoes contraction, regulating heart to pump blood, while diastole is defined as the period when heart undergoes a relaxation state allowing the filling of heart chambers and therefore continuing cycle of systole and diastole. The systolic and diastolic volumes of the heart are functions of atria and the ventricles. Stroke volume (SV) is defined as the volume of blood pumped from the left ventricle during the contraction (systole) and relaxation (diastole) state of the heart. Stroke volume is obtained by subtracting end diastolic volume from end systolic volume [21, 122]. Heart rate (HR) is calculated by the number of heart beats per minute. Cardiac output (CO) is defined as the amount of blood that is pumped out by the ventricle per minute, and is calculated by multiplying stroke volume and heart (SV*HR).

Stimulation of cardiomyocytes results in the opening of voltage gated calcium ion channels causing an influx of calcium ions from the extracellular matrix resulting in release of Ca^{2+} ions from ryanodine channels followed by muscle contraction at the heart. In addition to that, sarcoplasmic ATPase pump is responsible for resequestering Ca^{2+} back into the sarcoplasmic reticulum, thus causing muscle relaxation. These actions show the role of calcium ions in playing an important role in contraction and relaxation of heart muscle tissue. As these are among the aspects studied in this thesis research, we will briefly discuss the nervous and endocrine systems, and calcium binding proteins and their involvement in CV functions [21, 123-127].

1.3.1. Nervous Regulation of Cardiovascular Physiology

In vertebrates, the cardiac contraction, control of vascular resistance is mediated by the input from the autonomic nervous system (ANS; parasympathetic and sympathetic) [128, 129]. The ANS is comprised of sympathetic and parasympathetic systems [130, 131]. The sympathetic nervous system is initiated when body receives signals for emergency responses (flight or fight response). The cardiac sympathetic nervous system is mediated in the ANS when body undergoes stress [27, 130]. The preganglionic neurons arising from the sympathetic nervous system is localized at the upper thoracolumbar region of the spinal cord. The preganglionic fibres exit the spinal nerves through white rami branches followed by entry into the sympathetic ganglia. The cardiac neurons form the sympathetic ganglia are located along the visceral column (paravertebral ganglia with projecting into the postganglionic neurons). In order to mediate the activity of the sympathetic nervous system, sympathetic neurotransmitters are involved. Sympathetic neurotransmitters are substances that are released into the synaptic cleft in response to action potentials by transmitting signals from one neuronal cell to the target cell across the synaptic barrier. During this process, the preganglionic neurons of sympathetic and parasympathetic systems secrete ACh, referred to as the cholinergic system, while the postganglionic neurons of the sympathetic nervous system secretes norepinephrine (NE), released from the enterochromaffin cells. Adrenergic receptors are composed of two types: β (β_1, β_2 and β_3) and α (α_1 and α_2) adrenergic receptors [27]. Amongst the two adrenergic receptors, β_1 adrenergic receptors are predominant in the myocardium constituting to 75-80% when compared to β_2 (15-18%) and β_3 (2-3%) receptors [27]. β_1 adrenergic receptors are abundantly expressed in the SA node, AV node and cardiomyocytes while the β_2 adrenergic receptors are localized in smooth muscle and skeletal muscle. The role of β receptors increased heart rate, thereby

increasing contractility rate and intracellular Ca^{2+} release by the sarcoplasmic reticulum in response to norepinephrine and adrenaline.

The parasympathetic nervous system is generally responsible for the basal organ system function. The regulation of parasympathetic nervous system plays an antagonistic role in cardiac function [27]. The preganglionic neurons associated with the parasympathetic nervous system is expressed from the neurons located in the mid brain region and medulla oblongata [27, 132, 133]. The preganglionic fibers innervate the organs of the thorax and upper abdomen, which carries around 75% parasympathetic nerve fibres passing to the heart and other peripheral organs. These fibers synapse with the ganglion, and then reach the target organs via short postganglionic fibers [27, 128]. The predominant neurotransmitter of the parasympathetic nervous system is acetylcholine (ACh). ACh bind to two types of receptors namely, nicotinic and muscarinic receptors. Nicotinic receptors are expressed between the pre and post ganglionic synapses of the sympathetic and parasympathetic nervous system pathways [27, 132]. Neuromuscular junctions located between the skeletal muscle fibers are nicotinic and elicits rapid, excitatory response upon activation. Muscarinic receptors are expressed at the membrane ends of post-ganglionic parasympathetic nerves and cholinergic sympathetic nerve fibers and are classified into two types: M_2 and M_3 [27, 134, 135]. M_2 receptors are widely expressed in the heart tissue with abundant expression in nodal and atrial tissues in humans. Binding of M_2 receptors to ACh results in lowering heart rate, reducing conduction velocity via AV node. M_2 activation results in decreased contractility rates causing reduced stroke volume, heart rate and cardiac output in mammals [27]. M_3 receptors are expressed in vascular endothelial cells and its activation results in dilatation of the vessels and causes endothelium dependent release of

prostaglandins [132, 136, 137]. In contrast to the functions of sympathetic nervous system, parasympathetic nervous system has moderate effect on cardiac function. The parasympathetic system effects on cardiac function and contractility are negative chronotropic effect (lower heart rate), negative inotropic effect (reduced myocardial contractility) and negative dromotropic effect (decreased conduction velocity) [27]. How important are these systems in the regulation of cardiovascular system in fish?

A large number of studies have been performed to understand the autonomic control in teleost fish. The teleost heart receives a dual autonomic innervation from the adrenergic fibers and cholinergic fibers [128]. Adrenergic fibers are highly expressed in the teleost heart, reach the heart via vagus sympathetic trunk, along the coronary arteries and anterioral spinal nerves [128, 138, 139]. In mammals, the neuronal catecholamine is noradrenaline, when compared to both adrenaline and noradrenaline released by the adrenergic neurons in teleosts [128]. In addition to these, catecholamines bind to β-adrenoceptors within the myocardium exerting positive inotropic as well as chronotropic effects on teleost heart [128]. On the contrary, α-adrenoceptors have been shown to mediate a negative chronotropic effect on heart in perch and carp [140, 141]. In fish, the inhibition of these fibers is facilitated by the release of acetylcholine that binds to muscarinic receptors in the heart [139, 142, 143]. Heart rate of teleost fish is also affected by a tonic input by adrenergic and cholinergic control systems. The balance between the cholinergic and adrenergic tone of the heart plays an important role in heart rate variability, indicating the function of spinal autonomic and cranial cholinergic influence on the teleost heart [144].

The vasculature of the teleost fish heart is innervated by the adrenergic nerves, β and α adrenoceptors resulting in vasodilation [128]. In Atlantic cod, the administration of adrenergic nerve blocking agent, bretylium showed that the systemic adrenergic tone is dominated by the neural mechanisms of the autonomic nervous system over the circulating catecholamines [145-148]. However, few studies have shown that stressful stimuli to the fish might result in increased levels of catecholamines to increase the vascular resistance and arterial blood pressure [149]. On the contrary, several fish studies have reported the importance of the catecholamines in controlling the venous vasculature in teleosts [150]. Administration of adrenaline and noradrenaline increased central venous blood pressure and circulatory filling pressure *in vivo*, and it is partly mediated by adrenergic nerves in rainbow trout [151-153]. The control of the branchial vasculature in teleosts involves both adrenergic and circulating catecholamines and also cholinergic, serotonergic and neuropeptide-containing nerves [154, 155]. Adrenergic innervation projects via arterio-venous pathway where α-adrenoceptors dominate, and also in afferent filamental artery and the sphincter region wherein the β-adrenoceptors play an important role in assisting the adrenergic innervation of the branchial vasculature [139, 154, 155]. During this mechanism, the circulatory blood flow in the branchial vasculature favors the arterio-arterial pathway (respiratory) and restricts the blood flow from the arterio-venous side (nutritive) enabling the circulatory catecholamines to play an important role in control of the gill circulation than the systemic circulation [154, 155]. In addition to these, cholinergic receptors constrict the efferent filamental artery sphincter by binding to the muscarinic receptors in teleosts [154, 155].

In addition to its role on the control of cardiac function in teleost fish, a positive relationship between the nervous system and the gastrointestinal circulation in fish have been

studied [156]. Adrenaline increased the rate of vascular resistance in the GI tract of red Irish lord (*Hemilepidotus hemilepidotus*) [157]. In Atlantic cod, administration of vasoactive intestinal polypeptide (VIP) decreased the rate of resistance in the coeliac vascular bed whereas the mesenteric vascular resistance at the GI tract was unchanged [158]. The autonomic nervous system in fish during cardiovascular reflexes is controlled by both internal and external environments such as chemoreceptors (pH and oxygen levels), baroreceptors (vasculature walls) [139]. The activation of these receptors have shown to have a positive effect on the output of the autonomous nervous system in restoring the homeostasis in fish [139]. However, the role of these receptors is to affect blood pressure, hypoxic conditions and feeding in fish [139, 142]. Transient pre-branchial occlusion in fish resulted in tachycardia, increased aortic blood pressure and circulatory filling pressure, while post-branchial occlusion caused bradycardia [159]. In trout, pre-treatment with prazosin resulted in increased circulatory filling pressure and blood pressure during pre-branchial occlusion thereby stating the role of α adrenergic mechanisms in controlled arterial and smooth muscle tone [153, 159, 160]. Upon atropine administration, the chronotropic response was completely abolished showing that the modulation of vagal cholinergic tone was responsible for the cardiac function and alterations in blood pressure in trout [153, 159, 160]. Increased cholinergic tone reduced heart rate during hypoxia in teleosts and elasmobranchs [143, 147, 161, 162]. The reduction in the heart rate is entirely dependent on the magnitude of bradycardia. During hypoxia, there is an increase in the central venous blood pressure showing an active compensatory response in cardiac filling to increase the stroke volume in teleost fishes [159, 163, 164]. In addition to these, hypoxia increased the resistance in the gills of trout *in vivo* due to the increased cholinergic tone on the efferent filamental artery sphincter [165, 166].

Several studies have shown that feeding behaviour in fish have elevated the levels of the cardiovascular functions and gut blood flow [148, 156, 167, 168]. Gastric distension after a meal in rainbow trout (equivalent to 2% of body mass) and shorthorn sculpin (8-10% body weight) resulted in increased vascular resistance and dorsal aortic blood pressure [169, 170]. In addition to these, pre-ingestion of diet resulted in an increase cardiac output, stroke volume and GI blood flow in rainbow trout [170]. In conclusion, fish represents the most diverse group of the vertebrate family, where the autonomic nervous system plays an important role in controlling their cardio-respiratory system.

Numerous studies reported the mechanism of autonomous nervous system in cardiac functions by applying β-blockers. β-blockers are subdivided into first generation noncardioselective agents (propranolol, nadolol, oxprenolol, timolol and penbutolol) and cardioselective agents (atenolol, metoprolol, betaxolol, esmolol and nebivolol) [171]. Over the last decade, several researches have showed the importance of adrenergic blockers in the treatment of cardiovascular diseases. β-blockers have been widely used as drugs in the treatment of hypertension due to its ability in controlling the blood pressure under varying physiological conditions [172]. In addition to these, β-blockers, especially propranolol, have also been used in combination with vascular smooth muscle relaxation drugs such as hydralazine and dihydralazine during the treatment of hypertension [173]. β-blockers are also positive effectors in the treatment of angina pectoris (lack of adequate blood supply to heart muscle). The administration of β-blockers (propranolol, atenolol) have shown a positive effect in lowering the heart rate, reducing the myocardial oxygen demand and increasing the diastolic duration, resulting in increased coronary artery flow [174, 175]. Various studies have shown the positive

effect of bisoprolol, carvedilol, metaprolol and nebivolol causing reductions in morbidity and mortalities in chronic heart failure subjects [176-179]. In humans, β-blockers were widely used as drugs to treat post myocardial infarction and arrhythmias [180, 181]. In fish, administration of adrenaline elicited an increase in the arterial pressure resulting in bradycardia by activating the parasympathetic system which, could then be blocked by atropine [160, 182-184]. In zebrafish, exposure to propranolol (2.48 mg/L) and metoprolol (50 mg/L) decreased heart rate and increased apoptosis [185]. In trout, administration of BRL_{37344} (β_3- adrenergic receptor agonist) increased heart rate and reduced stroke volume, while administration of propranolol increased the dorsal aorta blood pressure [186]. In zebrafish and Japanese medaka, exposure of propranolol (0.1 µg/L) decreased heart rate and impaired cardiac improvement after 44 hour post fertilization (zebrafish) and at 68,116 and 164 hour post fertilization (Japanese medaka) [187].

1.3.2. Hormonal Regulation of Cardiovascular Functions

The regulation of cardiac function is also influenced by the action of hormones and naturally occurring ligands. The primary hormones that are released from the heart are atrial-natriuretic peptide (ANP) and brain-natriuretic peptide (BNP). ANP is secreted, stored and released by the atrial myocytes and are also expressed in the brain, renal glands and along the ventricular layers. The release of ANP from the heart is initiated upon β-adrenergic stimulation, increase in angiotensin II and endothelin levels, hypernatremia and distension from the atria respectively [188]. Physiological role of ANP is to reduce blood pressure via increased loss of Na in urine thereby reducing systemic vascular resistance and exhibits cardioprotective effects [189]. BNP on the other hand, is produced by ventricular cardiomyocytes and exhibit physiological roles similar to that of ANP in regulating cardiac function [190]. Other hormones/factors that modulate cardiac function include ghrelin, nesfatin-1 and glucagon like peptide-1 (GLP-1). Ghrelin, a gut hormone has been shown to play important roles in the regulation of cardiovascular, skeletal and immune system [191]. Administration of ghrelin resulted in improved cardiac systolic dysfunction in rats with heart failure [192]. Long-term exposure of ghrelin resulted in enhanced cardiac performance by stimulating cardiac output and stroke volume in rats [192]. Ghrelin also decreased the levels of norepinephrine and adrenaline thereby affecting the sympathetic nervous system and increasing the efficiency of the heart function [193], suggesting its role in regulating cardiovascular function in rabbits.

Nesfatin-1, an orphan ligand and anorexigenic peptide has also shown to be a key regulator of cardiac function in mammals. Administration of nesfatin-1 protected the myocardial tissue in rats treated with isoproterenol via AkT/GSK-3β dependent mechanism [194]. ICV

injections of nesfatin-1 increased plasma catecholamine levels, stimulated vasopressin and renin, and enhanced sympathetic activity [195]. In zebrafish, nesfatin-1 immunoreactivity (IR) was detected in the cardiomyocytes and administration of nesfatin-1 resulted in cardiosuppressive effect decreasing heart rate and cardiac output [21]. Glucagon like peptide-1 (GLP-1), another gut hormone is expressed in cardiomyocytes and smooth muscle in mice [18]. Administration of GLP-1 resulted in reduction of ischemia injury and improved contractility function, reduced blood pressure, and GLP-1 is expressed throughout the cardiovascular system in mice [18, 196, 197]. In eel, GLP enhanced the atrial beating and contractile force in a dose-dependent manner [198].

1.3.3. Calcium Handling and Muscle Proteins in Heart

Calcium handling is a critical aspect in cardiac function [199-201]. My research focused on two proteins that are shown to play important roles in calcium handling, SERCA2a and RyR1b. Sarco/Endoplasmic reticulum Ca^{2+} pump (SERCA), a calcium-handling pump is involved in muscle relaxation by the uptake of calcium ions. Contraction is mediated by the release of calcium with the help of ryanodine receptors, whereas the relaxation occurs by the uptake of calcium ions mediated by SERCA pump, stating that SERCA has the ability to restore calcium to maintain contraction-relaxation cycles. Three genes encoding SERCA, SERCA1, SERCA2 and SERCA3 have been identified in vertebrates [202-206]. The expression of SERCA1 is found in fast-twitching skeletal muscles, SERCA2 encodes SERCA2a, abundantly expressed in cardiac and slow twitching skeletal muscle, and SERCA2b found in many muscle at relatively lower levels [205, 207]. SERCA3 protein is abundant in non-muscular tissues. The physiological role of SERCA pumps has been widely studied in human and animal models in response to heart failure. Heart failure has been identified to have strong association with sarcoplasmic calcium uptake levels and intracellular calcium concentrations. The restoration of Ca^{2+} mediated transport system by increasing the expression of SERCA was found to be critical for maintaining cardiac functions [202]. In zebrafish, SERCA protein has been shown to play an important role in cardiac development and function. Another protein of interest is the ryanodine receptor 1b (RyR1b), which belongs to a family of intracellular Ca2+ channels that encodes sarcoplasmic reticulum known to regulate entry of calcium ions into the cytosol membrane from the intracellular organelles. Three RyR genes (RyR1, RyR2, RyR3) have been identified. RyR1 and RyR2 are primarily expressed in skeletal and cardiac muscle and RyR3 is expressed in other peripheral tissues. The physiological role of RyR is to stimulate the release of Ca2+ during

excitation and coupling. In zebrafish, RyR has been shown to regulate Ca2+ signals during embryonic development in zebrafish.

Muscle proteins also play a vital role in regulating cardiac development and function. Our research studied the effects of irisin and NLP in regulating muscle proteins including PGC-1 alpha, myostatin, troponin and tropomyosin. PGC-1 alpha, an exercise dependent hormone, is an important factor that helps muscle adaptation to endurance exercise [208]. In mice, deletion of PGC-1 alpha resulted in reduced muscle development and functionality and increased inflammation. Myostatin, a member of the transforming growth factor beta family is a secreted signalling mediator that plays an important role in suppressing the conversion of white adipose tissue to beige/brown adipose tissue in humans [209, 210]. In mice, deletion of myostatin resulted in increased cell mass, decreased body fat deposition, increased insulin sensitivity, increased fat oxidation and protection from obesity [211-213]. Deletion of myostatin increased the expression of AMPK, PGC1 alpha and FNDC5, leading to activation of browning of fat in mice [213]. Myostatins have shown to have a negative effect on satellite cell growth and postnatal myogenesis in zebrafish [214]. In rainbow trout, cortisol treatment elevated levels of myostatin-2a in muscle determining a positive relationship between the myostatins and cortisol levels in fish [215]. In addition to that, cortisol treatment elevated the levels of myostatin-1b mRNA in muscle of Brook trout and myostatin-2a mRNA expression in muscle of Atlantic salmon [216]. Troponin, a complex protein consisting of troponin C, troponin I and troponin T, is abundantly expressed in cardiac and skeletal muscle, and plays an important role in muscle contraction [217]. Cardiac troponin C is a primary determinant of cardiac contractility since it is a calcium binding protein that directly mediates responses to the amount of intracellular calcium

released in the heart [218]. On the other hand, cardiac troponin T is a key mediator protein that binds the troponin complex to tropomyosin to mediate controlled interaction between actin and myosin filaments in the myocardial cells of the heart [219]. Overexpression of troponin T has resulted in myocardial damage and its release into circulation from damaged cardiomyocytes is currently used as a biomarker for diagnosing acute myocardial infarction [19]. It is possible that myostatins and troponins contribute to the effects on cardiac and metabolic physiology in zebrafish. The list of endocrine factors regulating cardiovascular physiology and feeding is gradually growing.

1.4. NOVEL REGULATORS OF FEEDING AND CARDIOVASCULAR FUNCTIONS – FOCUS OF THIS THESIS RESEARCH

In vertebrates, the regulation of appetite, cardiac function, metabolism and homeostasis occurs through complex interactions of brain and peripheral signals [17, 120]. Neuropeptides and neurotransmitters located in the brain, mainly the hypothalamus regulate feeding and homeostasis, while the peripheral signals including adipokines and cytokines play a vital role in regulating homeostatic signalling pathways in mammals and vertebrates [55]. Hormones primarily act on the hypothalamus, known as metabolic centre of the brain, to regulate and modulate fish metabolism, cardiac function and feeding status [17]. What are the hormones that relay feeding status to the brain? Do these hormones have any role in cardiovascular physiology? These questions are still being addressed. In the last decade several novel peptide hormones [95] were discovered and various attempts were made to elucidate their physiological roles and mechanisms of action. While majority of these studies are done in mammals, limited information is also available from fish. Some of the naturally occurring hormone-like molecules of interest are nesfatin-1-like peptide (NLP) and irisin, which will be the focus of this thesis research.

1.4.1. NESFATIN-1-LIKE PEPTIDE

NLP is encoded in NUCB1. Nucleobindins (NUCB1 and NUCB2) are a class of multi-domain Ca2+ and DNA binding proteins that play an important role in cell signaling [220]. Nucleobindins are multifunctional proteins and are proposed as precursors of bioactive regulatory factors [220]. Human NUCB1 and NUCB2 are remarkably conserved (62% amino acid identity) within their bioactive regions (24-53 amino acids) [221-223]. In fact, NUCB2 was named so due to its high sequence similarity with NUCB1. In 2006, a novel anorexigen named nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-Influencing proteiN-1), an 82 amino acid anorexigenic peptide encoded in the N-terminal region of NUCB2 was reported [32]. NUCB2 is cleaved by prohormone convertases (PC 1/3 and 2), producing three peptide fragments, of which nesfatin-1 is the only one known to be biologically active [32]. NUCB1 is a 55 kDa protein [224]. NUCB1 was initially identified from a B-lymphocyte cell line from mice, and constitutes calcium and DNA binding motifs, leucine component, amino acid region and two EF hand. The leucine present in the NUCB1 is responsible for NUCB1 dimerization [222]. Williams et al., [35] reported that NUCB1 has been highly conserved in mammals and non-mammals.

1.4.1.1. TISSUE EXPRESSION OF NUCB1/NLP

Expression of NUCB1 has been reported in the pituitary lobes (anterior, posterior, intermediate) of mice by double-staining immunofluorescence [220]. NUCB1/NLP is abundantly expressed in the golgi region across all the cell types, endoplasmic reticulum, cytoplasm [35, 37]. Western Blot and immunofluorescence studies have shown that NUCB1 is localized in the pituitary, thyroid, gastrointestinal tract (GI tract), testis and gonads [225]. Immunogold staining

on the cryosections of ArT-20 and NRK cells and rat pituitary, liver and kidney reported that the localization of NUCB1 was concentrated on the cisternae and vesicles located on the cis side of the golgi stack [37, 226]. In addition to that, NUCB1 was strongly expressed in the endocrine pancreas along with insulin [35]. This cellular localization of NUCB1 suggests an insulin modulatory action [227]. In stomach, NUCB1 is abundantly expressed in the fundic gland region. NUCB1-IR was reported in the duodenum, jejunum and colon, especially concentrated in the duodenal enterocytes. NUCB1-IR was also detected in corticotrophs, somatotrophs, lactotrophs and gonadotrophs in the anterior pituitary. NUCB1 contributes in modulating matrix maturation in bone [228, 229]. Collectively, the localization of NUCB1 and its secretion from various endocrine organs suggest multiple roles for this protein in regulating various cellular processes.

1.4.1.2. NUCB1/NLP AND ITS FUNCTIONS

NUCB1 has shown to play an important role in regulation of $Ca2^{+}$ homeostasis, cell signaling and apoptosis [37]. NUCB1 is highly expressed at the site of Golgi and endoplasmic reticulum and has shown to increase cyclooxygenases-2 (COX-2) dependent prostaglandin E2 generation [36]. In mice, NUCB1 IR was detected in mouse MIN6 cells and pancreatic beta cells and possesses insulinotropic action *in vitro [227]*. In male Wistar rats, NLP (100 μg/kg B.W) reduced food intake and played an important role in modulating whole body energy balance [16].

1.4.2. IRISIN

Irisin is an exercise-induced 23 kDa myokine abundant in muscle [41] that is encoded in its precursor FNDC5. FNDC5 is a 212 amino acid protein, of which 32-138 amino acids correspond to irisin [46]. Upon endurance training, increased expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) mRNA is observed in skeletal and cardiac muscles [39, 230]. The increased expression of PGC1-α promotes FNDC5 mRNA, followed by the proteolytic cleavage of FNDC5 to release irisin from the skeletal or cardiac muscle into blood [230, 231]. In addition to skeletal and cardiac muscle, FNDC5 mRNA is also expressed tissues including rectum, brain, adipose tissue and pericardium [232, 233]. Irisin is abundantly expressed in skeletal muscle, cardiac muscle, adipocytes, brain, kidney, gonads respectively [41, 232, 234-236]. Irisin-IR was detected in the skeletal and cardiac muscle, and Purkinje cells in rodents by immunofluorescence studies [234, 237, 238]. In pigs, FNDC5 mRNA is strongly expressed in the skeletal muscle, and very low expressions in heart, liver and adipocytes. FNDC5 increased the proliferation of primary adipocytes, and enhanced the browning of white adipocytes in pigs [239]. Knockdown of FNDC5 decreased cardiac differentiation and downregulated mitochondrial integrity in mouse embryonic stem cells [240]. High protein diet and high fat diet composition modulated FNDC5 and irisin levels and increased brown adipose tissue mass in mice [241, 242]. Deletion of FNDC5 resulted in obesity, lowered insulin resistance, fat accumulation and inflammation in mice fed with high fat diet [243]. FNDC5 attenuated adipose tissue inflammation levels via AMPK-mediated macrophage polarization in high fat induced obese mice. Elevated levels of circulating irisin induced expression of thermogenin in white adipose cells, leading to browning of white adipocytes and increased thermogenesis [236]. It has been reported that overexpression of irisin increased

energy expenditure, reduced body weight, improved glucose tolerance and stimulated insulin resistance in high fat fed mice [39]. In zebrafish, irisin promoted angiogenesis through ERK signaling pathway [47]. In rodents, irisin has shown to play a significant role in regulating bone metabolism and enhance cognitive capacity [244]. Administration of irisin resulted in skeletal muscle hypertrophy and improved skeletal muscle strength in mice [245]. Only very limited information on NLP and irisin effects on feeding and cardiac functions in zebrafish and goldfish is available. My research will primarily focus on the role of nesfatin-1-like peptide and irisin effects on cardiovascular physiology and feeding in zebrafish and goldfish.

1.5. Fish as a Model Organism for Physiology Research

Fish is an important source of diet in most of the parts of the world. There has been a linear growth in aquaculture production with over 2 million tons yielded per year since 2010 [246, 247]. Fish represent the largest and most diverse class of vertebrates. Their ability to grow in adverse environments also makes them ideal for molecular, evolutionary, and neuroendocrine studies [248]. Easy handling, commercial availability, anatomical characteristics and relative ease of maintenance make fish as a desirable model for research. Taking these aspects into consideration, my research used teleosts (goldfish; *Carassius auratus*, and zebrafish, *Danio rerio,* two cyprinids) as model organisms to characterize naturally occurring novel peptides.

1.5.1. Goldfish

Goldfish, a freshwater fish belongs to Cyprinidae family has been used as an unconventional model organism to study a number of biological process. As a Cyprinid, goldfish is related to important ecological, genetic and/or cultured models including zebrafish and carp. Goldfish is considered extensively as a model to study neuroendocrine signalling [248, 249]. Most studies in goldfish focus on the interaction between brain and peripheral organs that regulate growth, reproduction, gonadal physiology and stress response. One of the main advantages of using goldfish is its ability to survive under laboratory conditions, ease of handling and maintenance and sample collection. Their natural habitat includes backwaters and streams equipped with aquatic vegetation. Goldfish thrive under pH levels of 5.5-7.0, high fluctuations of turbidity and water temperatures [248]. Adult goldfish can tolerate salinity levels up to 6 ppt and water temperatures up to 41° C. Goldfish can grow up to a maximum length of 20 cm and weight between 200-300g. Multiple sequential blood collection for performing various biochemical and

molecular experiments is feasible in goldfish. In addition to this, goldfish is equally suitable for performing *in vivo* and *in vitro* studies, such as growing primary cell cultures, integrative physiology, locomotor activity and behavioural studies [250-253].

Internal anatomy of goldfish is highly similar to other vertebrates, and consists of brain, pituitary, heart, GI tract, liver, spleen, kidney and gonads (testis and ovary). Our research mainly focused on brain, pituitary, heart and GI tract and their importance in studying integrative physiology and comparative endocrinology research. The brain of goldfish is very complex and highly organized into areas that play a vital role in regulating different physiological and behavioural functions [254]. One of the main advantages of the goldfish brain for the research use is its size, and this has led to many important contributions to fish research. Many hormones and their receptors including kisspeptin and neuromedin U (NMU) have been detected and identified from whole brain samples of goldfish [255]. Goldfish brain has been widely used for tissue culture studies on the neurophysiological and biochemical properties of brain, enzyme activity and its regulation. Goldfish brain has also served as a great model to conduct intracerebroventricular (I.C.V) injections and to determine the central effects of hormones and their physiological roles. Studies on neuroendocrine regulation of food intake, stress response, osmoregulation, behavioural functions and locomotor functions were conducted using goldfish as a model to compare and contrast the effects of hormones [77, 85, 95, 256, 257]. Numerous studies explored the internal organization of fish heart and its relationship to cardiac function. Based on shape and size of the goldfish heart, it has been used for studying cardiac regeneration and remodelling [258]. Studies related to survival and heart function under hypoxic/anoxic conditions have been widely performed using goldfish as a model organism [259]. Nesfatin-1, a

positive inotrope has been detected in goldfish heart has shown to play an important role in regulating cardiac performance, and cardiovascular protection in vertebrates [260].

1.5.2. Zebrafish

Zebrafish (*Danio rerio*) has been widely used as a research model to study metabolism, development and cardiovascular function [261-263]. Main advantages of zebrafish as research model are its small size, high fertility rate and cost efficiency. In addition to that, the whole genome information of zebrafish is available allowing to design genetic screens [120]. High throughput screening of zebrafish makes it a valuable model organism to study gene expression, pharmacological and drug testing studies. Due to its short generation times and production of high number of eggs, zebrafish are widely used model organisms to study chronic diseases, diseases of the immune system, metabolic disorders and cancer.

Numerous studies have been carried in zebrafish on stress responses, growth cycle and thyroidal system and how they are regulated by response of neuropeptides, pituitary hormones and other peripheral hormones. Zebrafish reproduction is very different to that of mammalian models having external fertilization and spawning rates at 100-200 eggs per week [264]. Similar to most fish, zebrafish growth cycle is very fast making it an ideal model for understanding hormonal regulation of growth [265]. Most importantly, the appetite regulatory mechanisms in zebrafish are highly conserved to mammals, making it an excellent model to study regulation and developmental systems involved in energy homeostasis throughout their life cycle [266].

One of the main advantages of zebrafish to study cardiovascular studies is the ability of their embryos to survive in the absence of cardiac function by oxygen diffusion from the water, unlike rodents that suffer hypoxic deterioration under similar condition [263, 267]. Zebrafish embryos develop externally and are transparent, allowing direct observation to study heart anatomy. Ultrasound monitoring has been very effectively used in zebrafish to study heart function [21, 122]. Some studies have also used zebrafish as a comparative model for muscle development studies [268]. Zebrafish was used to clone the first non-mammalian growth/ differentiation factor (GDF 11) which has been reported to play an important role in mesodermal formation and neurogenesis [269]. These key attributes of zebrafish embryos have made them an ideal model to understand cardiovascular development and function and other studies related to heart failure, and congenital heart diseases (CHD) [120].

1.6. RATIONALE

In rats, nesfatin-1, encoded in NUCB2 has been reported to have an inhibitory effect on feeding behavior and modulation of energy balance [270]. According to Werneke et al., [271], I.C.V injections of nesfatin-1 (25 pmol/rats) affected thermogenesis, thereby resulting in stimulatory effect on energy expenditure and lowering of food intake in rat. Nesfatin-1 has similar metabolic and reproductive functions in fish [95]. In addition to these effects, previous results from our lab have shown nesfatin-1 effects on cardiovascular physiology in zebrafish. NUCB1, especially the nesfatin-1 region within the NUCB1 are very highly conserved in fish and mammals. Does NLP regulate feeding? Does it modulate cardiovascular functions similar to that of nesfatin-1? If NLP regulates cardiac function, does it also modulate calcium-handling proteins expressed in the heart? My thesis research aims to address the above questions. Considering the high sequence conservation within the biologically active region of the peptide, it is likely that the NUCB1 encoded NLP is also biologically active.

Irisin, an exercise-induced myokine is released into the circulation by the processing of FNDC5 by PGC-1 alpha. Since its discovery in 2012, there are just over 500 articles on irisin and its physiological roles in mammals. Very limited information on irisin regarding its physiological roles in fish is available which will be focused in this research. FNDC5 mRNA is expressed in the brain, adipose tissue, gut and pericardium in humans [232]. Irisin was also detected in the cerebrospinal fluid and expressed in the hypothalamus, adipose tissue and skeletal muscle in humans [272, 273]. More recently, irisin has gained importance as a potential biomarker for myocardial infarction due to its abundance in cardiac muscle [39, 234]. Intracerebroventricular administration of irisin in rats resulted in increased blood pressure and

enhanced cardiac contractility [22, 274]. It has been reported that overexpression of irisin increased energy expenditure, reduced body weight, improved lipid metabolism and glucose tolerance, and suppressed insulin resistance in high fat fed mice [39, 275, 276]. Knockdown of FNDC5 suppressed neural differentiation of mouse embryonic stem cells in mouse [277]. In zebrafish, irisin promotes angiogenesis and modulates matrix metalloproteinase activity through the ERK signaling pathway [47]. Does irisin regulate feeding and modulate cardiac function in zebrafish? If irisin regulates cardiac function, what are the potential mechanisms of irisin action in mediating cardiovascular effects in zebrafish? Does irisin also regulate muscle proteins in zebrafish? These questions will be addressed to gain a deeper understanding of irisin in fish. The *overarching goal* of this thesis research was to characterize NLP and irisin in fish.

1.7. HYPOTHESIS AND SPECIFIC OBJECTIVES

The *central hypothesis* of this research was that NLP and irisin have diverse, semi-conserved, and tissue-specific effects in regulating feeding and cardiac physiology in fish.

The *specific objectives* of this research system were to:

1. Characterize NLP and test whether NLP is biologically active peptide in goldfish,

2. Test whether NLP regulates cardiovascular functions in zebrafish,

3. Determine whether irisin regulates feeding and modulates appetite regulatory peptides in zebrafish,

4. Study the role of irisin in cardiovascular physiology of zebrafish, and

5. Investigate whether the adrenergic system is involved in irisin regulation of cardiovascular effects in zebrafish.

The following chapters 2-6 details the research conducted to address the specific objectives listed above. The final chapter (7) provides a general discussion of cumulative findings, and considers some caveats of my research and future directions.

TRANSITION

The following chapter focuses on the first objective of my thesis: Nesfatin-1-Like Peptide Encoded in Nucleobindin-1 in Goldfish is a Novel Anorexigen Modulated by Sex Steroids, Macronutrients and Daily Rhythm. Nesfatin-1 is an 82 amino acid anorexigen encoded in a secreted precursor nucleobindin-2 (NUCB2). NUCB2 was named so due to its high sequence similarity with nucleobindin-1 (NUCB1). It was recently reported that NUCB1 encodes an insulinotropic nesfatin-1-like peptide (NLP) in mice. Here, we aimed to characterize NLP in fish. RT- qPCR showed NUCB1 expression in both central and peripheral tissues. Second, we determined whether NLP has any effects on food intake in fish.

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Contributions: L.S. planned and executed all studies except the ones cited later in this section, analyzed data, and prepared the manuscript draft. A.M.B. conducted the daily rhythm and diet studies, while J.I.B. and A.M.B. carried out the steroid studies. N.R. conducted the Western blot studies and antibody validation. L.F.C. provided the steroid silicon pellets and provided intellectual input in designing the steroid treatment. S.U. provided the original ideas, funding for this research, helped design experiments, assisted with *in vivo* studies, tissue sampling, data analysis and interpretation and manuscript preparation.

Chapter 2

Nesfatin-1-Like Peptide Encoded in Nucleobindin-1 in Goldfish is a Novel Anorexigen Modulated by Sex Steroids, Macronutrients and Daily Rhythm

2.1. Introduction

Nucleobindins (NUCB1 and NUCB2) are a class of multi-domain Ca^{2+} and DNA binding proteins that play an important role in cell signaling [220]. Nucleobindins are multifunctional proteins and are proposed as precursors of bioactive endocrine regulatory factors [220]. Human NUCB1 and NUCB2 are remarkably conserved (62% amino acid identity) within their bioactive regions (24-53 amino acids) [221-223]. In fact, NUCB2 was named so due to its high sequence similarity with NUCB1. In 2006, a novel anorexigen named nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-Influencing proteiN-1), an 82 amino acid anorexigenic peptide encoded in the N-terminal region of nucleobindin-2 (NUCB2) was reported [32]. NUCB2 is cleaved by prohormone convertases (PC 1/3 and 2) resulting in three peptide fragments, of which nesfatin-1 is the only one known to be biologically active [32]. Administration of the bioactive core (M30, mid-segment 30 amino acids) of nesfatin-1 inhibits food intake and reduces body weight in rodents [32, 278]. In rats, nesfatin-1 inhibits feeding and promotes energy expenditure [279]. In mice, nesfatin-1 secretion is modulated by nutrients suggesting that nesfatin-1 play an important role in metabolism and energy homeostasis [33]. Administration of nesfatin-1 (25 pmol/rat) affected thermogenesis resulting in stimulation of energy expenditure and lowering of food

intake in rats [271]. In goldfish, nesfatin-1 reduces feeding activity [95, 280] and reproductive hormone secretion [95, 280, 281]. Nesfatin-1 was also detected in zebrafish [282], Ya fish [96] and trout [283]. Nesfatin-1 is now considered a multifunctional peptide in fish and mammals.

More recently, NUCB1 gained attention due to its similarity with NUCB2 and nesfatin-1. For example, our *in silico* analysis found that NUCB1 in fish and mammals encode a nesfatin-1 like sequence [220, 227], and these peptides possess prohormone convertase sites that enable its processing [227]. Immunofluorescence studies also revealed that the localization of NUCB1 is highly concentrated in islet cells in mice [227]. NUCB1 is very highly conserved in mammals and non-mammals. Our lab, for the first time, reported the discovery of a nesfatin-1-like peptide (NLP) in mice and its insulinotropic actions on mice pancreatic beta cells [227]. Whether NLP has appetite regulatory roles remain unknown.

This research aimed to determine two important aspects of NUCB1/NLP in goldfish, a well-characterized model in neuroendocrinology research. The first topic addressed was the tissue specific expression, and regulation of endogenous NUCB1 in goldfish. Second, we determined whether NLP has any effects on food intake in fish. Our results show tissue abundance and cell specific expression of NUCB1/NLP. This research also provides the novel evidence for daily rhythmic pattern under light: dark cycle, steroid, energy status and macronutrient modulation of NUCB1 mRNA expression in goldfish. Finally, we report the discovery of an anorexigenic activity for NLP.

2.2. Materials and Methods

2.2.1. Animals

Goldfish (*Carassius auratus*, common variety) were purchased from Aquatic Imports (Calgary, Canada). Goldfish (4-5 inches long, body weight: 25 g) were maintained at 24° C under 12L:12D photoperiod cycle. Unless otherwise specified, fish were fed once daily with a 4% body weight ratio at 11 AM with slow sinking pellets (slow-sinking pellets; Aqueon®, Catalog # 06053). Euthanasia was conducted using 0.5% tricaine methanesulfonate (TMS-222; Syndel Laboratories, Catalog # 5980A) followed by spinal transection. All animal experimentations complied with the policies of the Canadian Council for Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board (2012-0033).

2.2.2. In Silico Analysis

NUCB1 sequences from various species were obtained from GenBank (http://www-ncbi-nlm-nih-gov.cyber.usask.ca/nuccore/) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The signal peptide site in the zebrafish NUCB1 sequence was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The GenBank Accession numbers of sequences used are provided in figure legends.

2.2.3. Real-time Quantitative PCR (RT-qPCR), Qualitative PCR and Gel Electrophoresis

` Samples of brain, anterior intestine (J-loop region), midgut, rectum, eye, liver, heart, muscle, gill, testis, ovary and skin were collected from mixed population of goldfish (female and male) in order to study the tissue distribution of NUCB1 (n = 6). Fish were anesthetized using

TMS-222 before dissection. Tissue samples were collected for total RNA extraction and were stored in −80 °C. Total RNA was extracted using TRIzol RNA extraction reagent (Catalog # 15596-026, Invitrogen). cDNAs were synthesized using iScript cDNA reverse transcription supermix (Bio-Rad Laboratories, Catalog # 170-8841) (**Table 2.1**). The cDNAs were used for RT-qPCR. The reaction protocol was setup with CFX connect real time PCR detection system (Bio-Rad,Canada) as per **Table 2.2**. The primers were validated and optimized for efficiency and annealing temperatures. For tissue distribution, quantitative PCR were conducted and run on a thermal cycler (Bio-Rad, Canada). The components for the qualitative PCR master mix and reaction components for running qualitative PCR are provided in **Table 2.3 and 2.4**. Real-time quantitative PCR was carried out using iQ SYBR Green supermix (Bio-Rad, Catalog # 170-8880) (**Table 2.1**) and CFX Connect Optics module system (Bio-Rad, Canada) controlled by CFX Connect PC-based software (Bio-Rad, Canada) (**Table 2.2**) and was analyzed using the Livak method described earlier [280]. Relative mRNA expression of genes of interest were quantified and normalized to the expression of elongation factor 1α (EF1α) (tissue distribution), beta actin (diet study, daily rhythms) and 18s RNA (food deprivation, estradiol and testosterone treatment and food intake studies) were used as housekeeping genes in the respective studies performed during the research (**Table 2.5**). The internal control gene was chosen based on the gene that provided the most consistent Ct values provided in each study.

Table 2.1. Components for RT-qPCR set up

Components	Volume Per Reaction
iQ™ SYBR® Green Supermix	5 μL
Forward Primer	0.5 μL
Reverse Primer	0.5 μL
cDNA	2 μL
Millipore Water	2 μL
Total Volume of the Sample	10 μL

Table 2.2. RT-qPCR run set up in CFX connect system

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	95 °C	95 °C	Annealing Temperature	95 °C	95 °C
Time	2 min	10 seconds	30 seconds	30 seconds	Infinite Hold

Table 2.3. Components for PCR master mix for qualitative PCR

Components	Volume
10X PCR Buffer Reaction Mixture	100 μL
25 nm $MgCl_2$	60 μL
dATP	5 μL
dTTP	5 μL
dCTP	5 μL
dGTP	5 μL
Millipore Water	820 μL
Total Volume	1000 μL

Table 2.4. Reaction components for qualitative PCR

Components	**Volume**
PCR Master Mix	20 μL
Forward Primer	1 μL
Reverse Primer	1 μL
cDNA Template	2 μL
Taq Polymerase	0.5 μL
Total Volume	24.5 μL

Table 2.5. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in goldfish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Gene Bank Accession Number
	Forward	Reverse		
NUCB1	CTGTCTCTGTGT CTGCTGGT	TGGTGCTGTCCAG TTTAGCC	60	KU903286.1
β-Actin	CTACTGGTA TTGTGATGGACT	TCCAGACAGAG TATTTGCGCT	59	AF057040.1
18s	GGATGCCCT TAACTGGGTGT	CTAGCGGCGC AATACGAATG	60	KY486501.1
Preproghrelin	ATTCAGAGTG TTGTCGTA	AGGAAAGAGC ACATAAGA	56.6	[280]
Orexin-A	GCATATCGG CCGCTTTAATA	GGGTCCTCGAG TCTCTTTCC	60	[280]
CART	GTGCCGAGAT GGACTTTGAC	AGCTGCTTCTC GTTGGTCAG	60	NM_001017570.1
EF-1α	CCCTGGCCACAGA GATTTCA	CAGCCTCGAACTC ACCAACA	60	NM_131263.1

2.2.4. Western Blot Analyses

Goldfish brain, pituitary, gut, ovary and testis samples were collected to confirm the presence of NUCB1 by Western blot analysis, conducted as described earlier [227]. Fish (n = 4) were anesthetized using 0.5% TMS-222 before dissection. Tissues for Western blot were homogenized using T-PER tissue protein extraction reagent (Catalog # 78510, Thermo Scientific, Vantaa, Finland) followed by protein concentration determination by Bradford assay using NanoDrop 2000c (Thermo). The samples were prepared using 1X Laemmli buffer containing 0.2% of 2-mercaptoethanol (Bio-Rad, Catalog # 161-0737 and -0710) and the samples were subjected to boiling at 95 °C for 5 min followed by vortexing prior to loading. The whole sample volume (30 µL), each containing 40 µg protein was electrophoresed on a Mini-PROTEAN TGX 8–16% gradient gel (Bio-Rad, Catalog # 456-1104) at 200 V for 20–30 min. After the run, the proteins were transferred to a 0.2 µm BioTrace nitrocellulose membrane (PALL Life Sciences, Catalog # 27377-000) subjected to blocking using 1X RapidBlock solution (aMRESCO, Catalog # M325). In order to detect the presence of NUCB1 and vinculin (reference) protein, rabbit polyclonal anti-mouse nucleobindin-1 (custom synthesized, Catalog # 1312- PAC- 02, 1:3000, Pacific immunology, Ramona, CA) for NUCB1 and rabbit polyclonal anti-vinculin (Catalog # ab73412, 1:1000, Abcam, Massachusetts) for vinculin were used. As secondary antibody, goat anti-rabbit IgG (H + L) HRP conjugate (Catalog # 170-6515, 1:3000, Bio-Rad) was used. For visualization of protein, the membrane was incubated for 5 min in Clarity Western ECL substrate (Bio-Rad, Catalog # 170-5061) and imaged using ChemiDoc MP imaging system (Bio-Rad, Catalog # 170-8280). Stripping for detection of reference protein in the membrane was conducted using western blot stripping buffer (Thermo Scientific, Catalog # 46430). Primary antibody was pre-absorbed in 3.33 µg synthetic goldfish/zebrafish NLP (Pacific

immunology, Ramona, CA) overnight and was used as pre-absorption controls for goldfish tissues to confirm the specificity of the NUCB1 antibody. Precision plus protein dual Xtra standards (Bio-Rad, Catalog # 161-0377) were used as markers to detect the molecular weight of protein of interest (NUCB1, preabsorption and vinculin). The NUCB1 antibody used in this study detects both the precursor NUCB1 and processed NLP.

2.2.5. Immunohistochemistry

The presence and localization of the protein in different tissue sections of goldfish were detected by immunohistochemical (IHC) studies. Zebrafish were anesthetized with TMS-222 (0.5%) and pituitary, ovary, J-loop and testis were collected from goldfish and collected in 4% paraformaldehyde. After a 24 hour incubation period, the fixed tissues were washed with 70% ethanol and embedded in paraffin and sent to sectioning at Prairie Diagnostic Services at University of Saskatchewan. Following the sectioning, the slides were deparaffinized with xylene (3 x 10 min) and rehydrated in series of graded ethanol (100%, 95%, 70% and 50%, 2 min) prior to incubation with primary antibody incubation . The antibodies used were: rabbit polyclonal anti- mouse nucleobindin-1 (Pacific immunology, Ramona, CA) for NUCB1, mouse monoclonal anti-growth hormone (Catalog # CLX 130AP, 1:500, Cedarlane, USA) for growth hormone in pituitary, mouse monoclonal anti-ghrelin hormone (Catalog # ab57222, 1:500, Abcam, Massachusetts) for ghrelin in J-Loop [280], mouse monoclonal anti-SOX9 hormone (Catalog # ab76997, 1:500, Abcam, Massachusetts) for SOX9 in testis [284, 285], goat monoclonal anti-FOXL2 hormone (Catalog # PA5-18175, 1:500, Waltham, MA) for FOXL2 [284, 285] staining in ovary respectively. The ghrelin antibody was previously validated for use in goldfish [280]. Both NUCB1 and GH antibodies were validated in this research using

preabsorption controls. SOX9 and FOXL2 antibodies were raised against an epitope that has approximately 85% similarity with zebrafish SOX9 and FOXL2. The slides were then washed with PBS and then were incubated with secondary antibody for one hour at room temperature. Goat polyclonal anti-rabbit IgG (Catalog # TI-1000, 1:500 dilution, Vector Laboratories, California) for NUCB1, goat polyclonal anti-mouse IgG H&L (FITC green- Growth Hormone, ghrelin, SOX9, Catalog # ab6785, 1:500 dilution, Abcam, Massachusetts) and Donkey Anti-Goat IgG H&L (Alexa Fluor 488, FOXL2; Catalog # ab150129, 1:500 dilution, Abcam, Massachusetts) were used as secondary antibodies. The slides were then rewashed with PBS and were mounted on Vectashield medium containing DAPI dye (Blue, Vector Laboratories). The slides were dried and imaged using a Nikon inverted microscope (L100) (Nikon DS-Qi1 MC camera, ON, Canada) and analyzed using NiS Elements imaging software (Nikon, Canada). Slides incubated with secondary antibody alone, or preabsorption [280] using synthetic goldfish NLP were used as negative controls. Since the antibody used here detects both NUCB1 and NLP, we used the term NUCB1/NLP-like immunoreactivity to refer to the staining obtained in our immunohistochemical studies.

2.2.6. NUCB1 Tissue Expression Profile in Goldfish

Goldfish (n = 6 fish/group) were maintained and fed daily at Zeitgeber time- 4 (ZT-4) as described earlier [286]. On the day of study, fish (mixed population) were collected randomly, and sampling was done at 4 hour intervals throughout the 24 hour cycle period starting from ZT-0 (light phase) until ZT-24 (dark phase). On the day of experiment fish were fed at ZT-4 respectively. Upon euthanasia, fish were dissected and hypothalamus, gut, hindbrain, forebrain and pituitary were collected and stored at −80 °C until total RNA extraction. Sampling of tissues

during the dark phase was done under dim red lighting. To study the relative mRNA expression of NUCB1, RT-qPCR studies were carried out as described earlier on hypothalamus, gut, forebrain, hindbrain and pituitary of goldfish.

2.2.7. *In Vivo* Diet Study

Male and female goldfish were grouped and weight matched (n = 8/group), and five groups of fish were fed with five different diets (custom diet containing high carbohydrate, high protein, high fat (9%), very high fat (20%) and control) for 1 and 4 weeks. The details of diet composition are provided in Blanco et al.[256]. The calorie content of the respective diets were: control (Test Diet, Catalog # 8887) 3.43 kcal/g2 with 37.6% energy derived from protein, 46.6% energy derived from carbohydrate and 15.8% energy derived from fat; high carbohydrate (Test Diet, Catalog # 8890) 3.87 kcal/g2 with 29.2% energy derived from protein, 56.8% energy derived from carbohydrate and 14% energy derived from fat; high protein (Test Diet, Catalog # 8893) 3.59 kcal/g2 with 50.4% energy derived from protein, 44.5% energy derived from carbohydrate and 5.1% energy derived from fat; high fat (9%) (Test Diet, Catalog # 8889) 3.61 kcal/g2 with 35% energy derived from protein, 49.9% energy derived from carbohydrate and 15.1% energy derived from fat; very high fat (20%) (Test Diet, Catalog # 8886) 4.27 kcal/g2 with 30.3% energy derived from protein, 27.6% energy derived from carbohydrate and 42.1% energy derived from fat. Hypothalamus and gut were collected upon euthanasia, followed by measurement of NUCB1 mRNA expression relative to the expression of beta-actin as reference gene.

2.2.8. In Vivo Food Deprivation Studies

In this study, the expression of NUCB1 mRNA was determined upon food deprivation in goldfish (both males and females pooled together). The relative expression of NUCB1 was determined in goldfish hypothalamus and gut after 3 and 7 days of food deprivation (n = 6/group). Fish in the control group were fed as usual. On the sampling day (3 or 7 days), during 11 AM–12 PM, goldfish from the fed and unfed cohorts were euthanized using 0.5% TMS-222 post 1 hour of feeding time. Subsequently, the hypothalamus and gut tissues were collected and stored at −80 °C for total RNA extraction followed by measurement of NUCB1 mRNA expression relative to expression of 18 s RNA housekeeping gene.

2.2.9. In vivo Treatment of Goldfish with Estradiol and Testosterone

For this study, female goldfish (n = 7/tank) were maintained as described earlier. On the day of experiment, solid silicone pellets containing estradiol or testosterone were prepared and washed thoroughly in saline and implanted intraperitoneally. Details of this study were previously validated in our lab [287]. Three different doses 25, 50 and 100 μg/g B.W were initially considered and compared to no treatment group (control). We found that 100 μg/g B.W of estradiol or testosterone was effective in elevating steroid hormone levels in goldfish and this dose was used to study the NUCB1 mRNA expression in goldfish. After 2.5 days of implantation, fish were euthanized and forebrain, hindbrain, gut, hypothalamus, pituitary were collected and NUCB1 relative mRNA were quantified using RT-qPCR.

2.2.10. Effect of Exogenous NLP on Feed intake and Appetite Regulatory Peptides in Goldfish

Fish (n = 6/group in each study) were maintained as described earlier. Synthetic rat and goldfish/zebrafish NLP were intraperitoneally injected (200 microliters) at four different doses i.e. 0, 0.1, 1, 10 and 100 ng/g B.W just prior to their scheduled feeding time (11 AM). Goldfish/Zebrafish NLP (VPIDRNPDPPQEEKAEENVDTGLYYDRYLREVIEVLETDPHFREKLQTANTEDIKNGRL SKELDLVGHHVRTRLDEL, Ramona, CA, >95% purity) was synthesized by Pacific immunology (Ramona, CA), and synthetic rat NLP (VPVDRAAPHQEDNQATETPDTGLYYHRYLQEVINVLETDGHFREKLQAANAEDIKSGK LSQELDFVSHNVRTKLDEL) was synthesized by Abgent Technologies, California with >95% purity. A scrambled peptide (79 amino acids) was designed using the Sequence Manipulation Suite™ online tool (www.bioinformatics.org/sms2/). NLP Scramble peptide (PDSRSDDGSPSVQLQDYALIADAEVTLTHIELFGSPQNATKLLNKTERLRFLKVVRGKH RENVVATEHYQAQKYPEEDE) with the lowest similarity to the rat NLP sequence was selected. The peptide synthesized was >95% pure (Pacific Immunology Corp, California, USA) and the mass and purity were confirmed by LC-MS. The control group (n = 6) were injected with 0.9% sodium chloride. Immediately after NLP administration fish were allowed to recover and were briefly fed and the feed was recovered post 1 hour (dried overnight at 60 °C) to quantify feed intake. In the time course study, food was recovered at 1, 2 or 4 hours post-injection. Goldfish euthanasia, tissue collection and processing and RT-qPCR were conducted as described earlier. Tissues collected were used for studying the expression of appetite regulatory peptides

preproghrelin, orexin-A and CART mRNAs. Data were normalized to 18 s RNA (housekeeping gene).

2.2.11. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using PRISM version 5 (GraphPad Inc., USA) and IBM SPSS version 21 (IBM, USA) were used for statistical analysis. $P < 0.05$ was considered statistically significant. Data are represented as mean + SEM. For daily rhythmicity of NUCB1/NLP gene expression, cosinor analysis was used by fitting periodic sinusoidal values in relevance to the expression values of the gene of interest for the seven time points taken into consideration during the study. Cosinor analysis was performed using the formula $f(t) = M + A\cos(t\pi/12 - \phi)$, where f(t) is the gene expression level in a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours and ϕ is the acrophase (time of peak expression). Significance of cosinor analysis was analyzed using the zero-amplitude test, which specifies that if sinusoidal amplitude differs from 0 with a given probability during the 24 hour profile. The time series data were plotted to display a 24-h rhythmic pattern with cosinor analysis.

2.3. Results

2.3.1. In Silico Analysis of NUCB1 Sequences

Sequence analysis found a very highly conserved nesfatin-1-like peptide (**Figure 2.1 a**) encoded in goldfish NUCB1 (GenBank Accession # KU903286). Goldfish NLP is identical to zebrafish NLP (**Figure 2.1 a**). The proposed bioactive core (M30) of NLP (77 amino acids) is very highly conserved across species. Goldfish/zebrafish NLP exhibits 74% amino acid sequence identity with zebrafish/goldfish nesfatin-1. A signal peptide cleavage site was predicted at positions 19 (Arginine) and 20 (Valine) in zebrafish and goldfish NLP sequences. Phylogenetic analysis found clustering of goldfish NUCB1 with NUCB1 from other fishes (**Figure 2.1 b**).

2.3.2. Tissue Distribution of NUCB1 in Goldfish

Abundance of NUCB1 mRNA expression was detected in several tissues including the hypothalamus, midbrain, hindbrain, muscle, pituitary, heart, olfactory bulbs and ovary (n = 6 goldfish). The expression of NUCB1 mRNA was normalized to EF-1α, which served as a reference gene to verify the quality and amount of goldfish mRNA samples (**Figure 2.1 c**). Western blot analysis detected NUCB1 at 55 kDa in goldfish tissue samples (**Figure 2.1 d**). No bands of expected size representing NUCB1 or NLP were detected in the pre-absorption control (Figure 1e). Vinculin (125 kDa) was used as a reference protein (**Figure 2.1 f**). The NUCB1 antibody used here only detected NLP, but not nesfatin-1 (**Figure 2.2**). In addition, pre-absorption of this antibody using synthetic goldfish/zebrafish NLP resulted in no staining for NLP (**Figure 2.2**). One-way ANOVA followed by Tukey's multiple comparison test (F value – 32.37; P – value – 0.034) were used for statistical analysis.

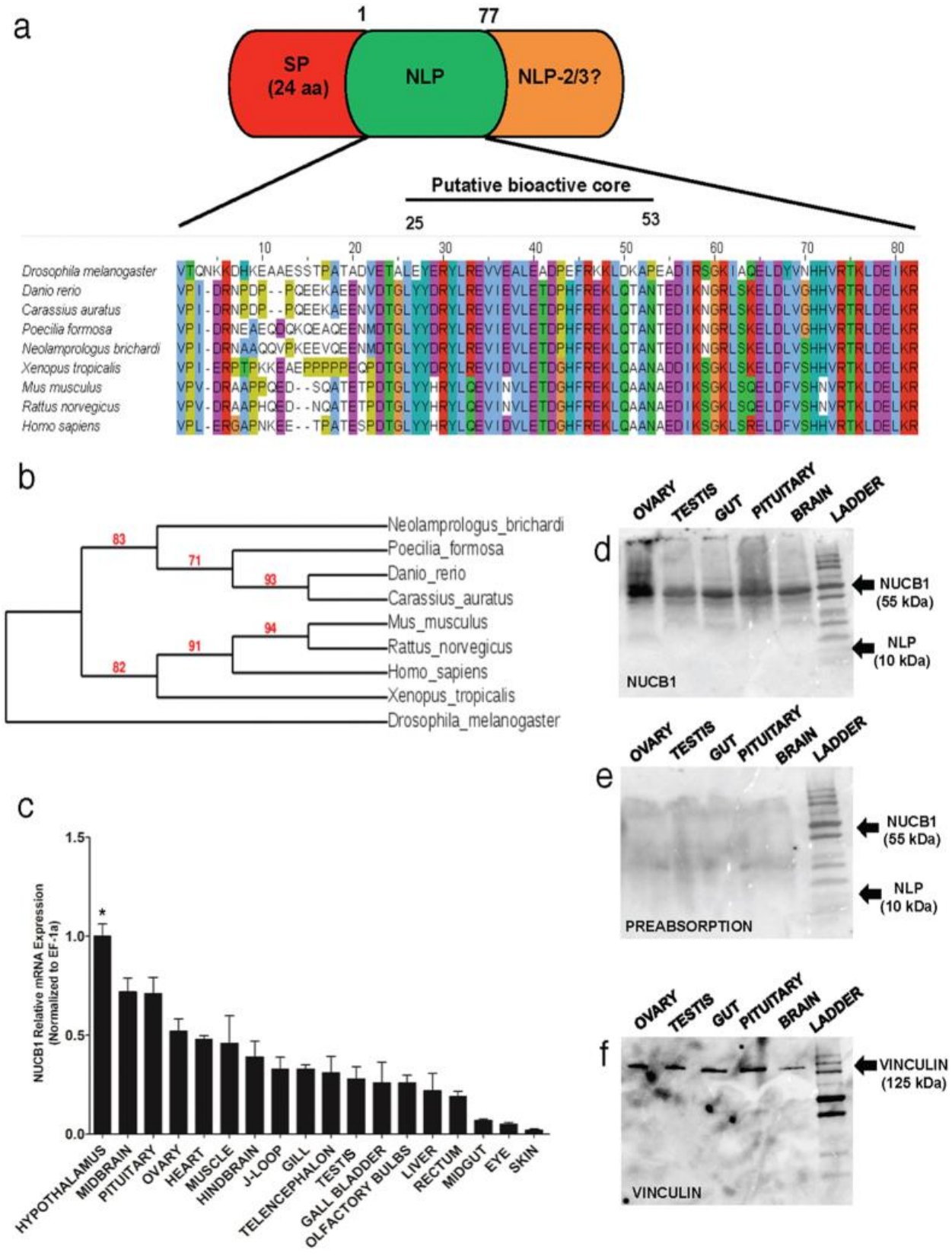

a
1
77
SP
(24 aa)
NLP
NLP-2/3?
Putative bioactive core
25
53
Drosophila melanogaster
Danio rerio
Carassius auratus
Poecilia formosa
Neolamprologus brichardi
Xenopus tropicalis
Mus musculus
Rattus norvegicus
Homo sapiens
b
Neolamprologus_brichardi
Poecilia_formosa
Danio_rerio
Carassius_auratus
Mus_musculus
Rattus_norvegicus
Homo_sapiens
Xenopus_tropicalis
Drosophila_melanogaster
83
71
93
94
91
82
c
NUCB1 Relative mRNA Expression
(Normalized to EF-1a)
HYPOTHALAMUS
MIDBRAIN
PITUITARY
OVARY
HEART
MUSCLE
HINDBRAIN
J-LOOP
GILL
TELENCEPHALON
TESTIS
GALL BLADDER
OLFACTORY BULBS
LIVER
RECTUM
MIDGUT
EYE
SKIN
d
OVARY
TESTIS
GUT
PITUITARY
BRAIN
LADDER
NUCB1
(55 kDa)
NLP
(10 kDa)
NUCB1
e
PREABSORPTION
f
VINCULIN
(125 kDa)
VINCULIN

Figure 2.1. NUCB1 is abundantly expressed in hypothalamus of goldfish. (**a**) Schematic representation of the NUCB1 precursor showing the signal peptide, and nesfatin-1-like peptide (1–77 amino acids) regions. Nesfatin-1-Like Peptide. 2/3 is referred as NLP 2/3. The alignment of NLP sequences from various species is shown underneath. Phylogenetic analysis of nucleobindin-1 gene sequences of various species is shown in (**b**). NUCB1 sequences consisting of signal peptide (1–24 amino acids) and the putative bioactive core (24–53 amino acids) were used for generating the cladogram. GenBank accession numbers of sequences used are: Carassius auratus (KU903286), Neolamprologus brichardi* (XM_006803054.1), Drosophila melanogaster (NM_140751.4), Poecilia formosa* (XM_007562129.1), Xenopus tropicalis (NM_213689.2) Danio rerio (NM_001045463.1), Mus musculus (NM_001163662.1), Rattus norvegicus (NM_053463.1), Homo sapiens (NM_006184.5). (**c**) The data was quantified using RT-qPCR (Real-Time - Quantitative PCR) in goldfish. The mRNA expression was normalized to elongation factor (EF)-1α ($n = 6$ goldfish). Western blot showing NUCB1 in brain, pituitary, gut, ovary and testis (**d**), preabsorption control in tissues tested (**e**), and vinculin (**f**) ($n = 4$ goldfish, representative blot is shown). Asterisk denotes significant differences between hypothalamus and other tissues ($p < 0.05$). Data are represented as mean + SEM.

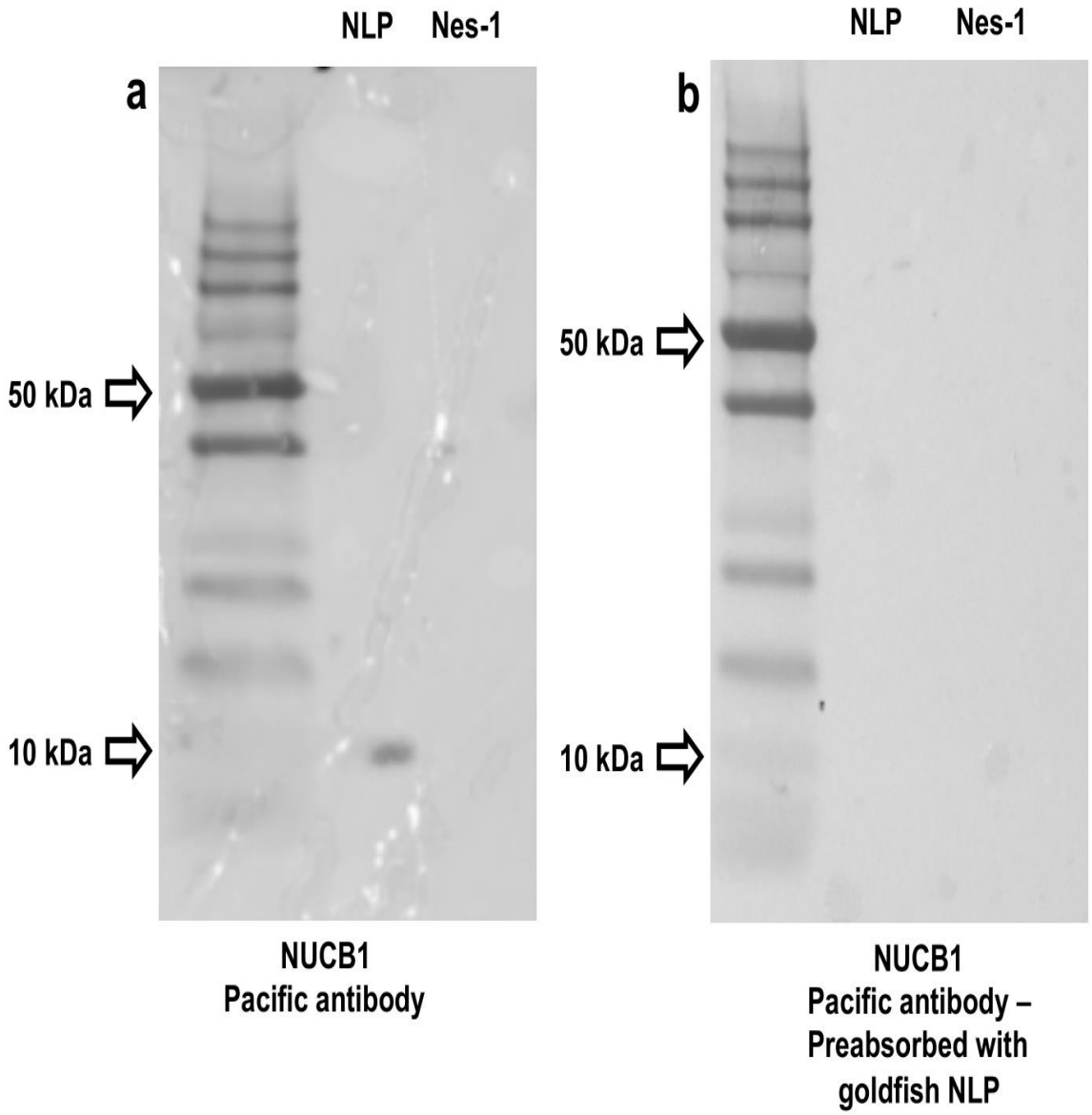

NLP
Nes-1
a
50 kDa
10 kDa
NUCB1
Pacific antibody
NLP
Nes-1
b
50 kDa
10 kDa
NUCB1
Pacific antibody –
Preabsorbed with
goldfish NLP

Figure 2.2. Western blot analysis showing the specificity of the NUCB1 antibody used for Western blot analysis and immunohistochemistry. In figure a below, it is shown that the antibody detects only synthetic NLP, but not nesfatin-1. In the figure b, the NLP staining disappeared when the antibody was pre-absorbed with goldfish synthetic NLP.

2.3.3. Cell Specific NUCB1/NLP-like Immunoreactivity in Gut, Pituitary, Testis and Ovary

NUCB1/NLP-like immunoreactivity (red) was found in goldfish J-Loop (**Figure 2.3 b**), testis (**Figure 2.3 f**), ovary (**Figure 2.3 j**) and rostral pars distalis (RPD) region of pituitary (**Figure 2.3 o**). Ghrelin (**Figure 2.3 a**), SOX9, Leydig cell marker (**Figure 2.3 e**), Forkhead box L2 (FOXL2), a thecal/follicular cell marker (**Figure 2.3 i**) and growth hormone (**Figure 2.3 m**) were used as cell specific markers. In the gut, cells in the villi were positive for both NUCB1/NLP and ghrelin (**Figure 2.3 c**). Leydig cell cytoplasm in goldfish testis co-localized NUCB1/NLP and SOX9 (**Figure 2.3 g**). Thecal and/or follicular cells within the goldfish ovary were stained positive for NUCB1/NLP and FOXL2 (**Figure 2.3 k**). DAPI (blue) stained the nuclei of cells. No immunoreactivity was observed in preabsorption controls (**Figure 2.3 n–GH, 2.3-d,h,l,p** – **NUCB1**).

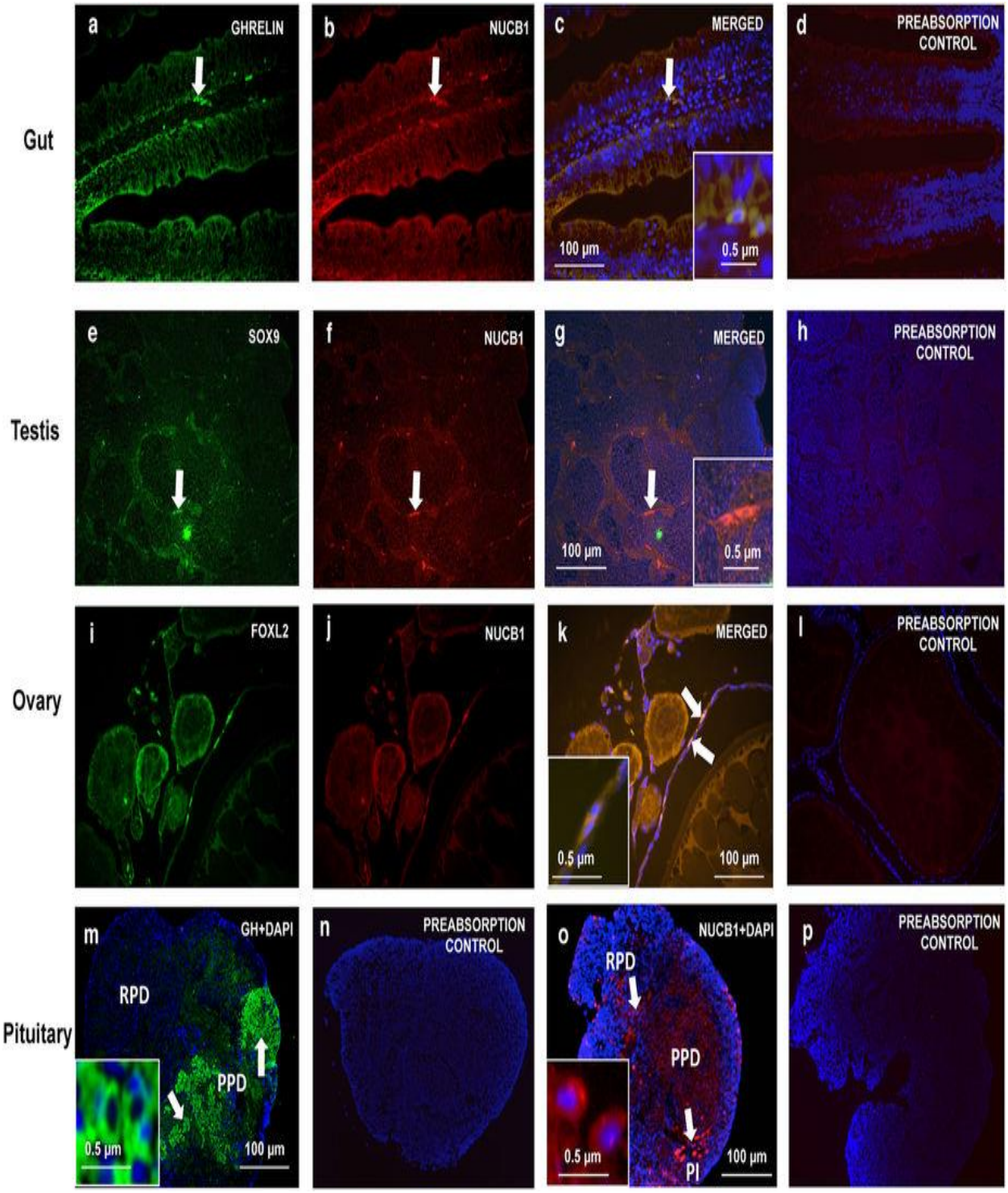

Gut
a
GHRELIN
b
NUCB1
c
MERGED
d
PREABSORPTION CONTROL
100 µm
0.5 µm
Testis
e
SOX9
f
NUCB1
g
MERGED
h
PREABSORPTION CONTROL
100 µm
0.5 µm
Ovary
i
FOXL2
j
NUCB1
k
MERGED
l
PREABSORPTION CONTROL
0.5 µm
100 µm
Pituitary
m
GH+DAPI
RPD
PPD
0.5 µm
100 µm
n
PREABSORPTION CONTROL
o
NUCB1+DAPI
RPD
PPD
PI
0.5 µm
100 µm
p
PREABSORPTION CONTROL

Figure 2.3. Cell Specific NUCB1/NLP-like immunoreactivity in gut, pituitary, testis and ovary. Immunofluorescence representing NUCB1/NLP immunoreactivity (Red; Texas Red; and in Green are Ghrelin (J-Loop); SOX9 (Testis); FOXL2 (Ovary); Growth Hormone (Pituitary) in the J-Loop (**a–c**), testis (**e–g**) and ovary (**i–k**) and pituitary (m–p) of goldfish. Nuclei are stained blue (DAPI). Representative cells showing immunoreactivity in goldfish tissues are marked with arrows. A magnified image of representative NUCB1/NLP-ir cell is shown in square inset in figure (**c,g,k,o**) and GH (n). Images were taken at 40X magnification and scale bar = 100 μm (and 0.5 μm for inset).

2.3.4. High Carbohydrate and High Fat Diet Decreases NUCB1 mRNA Expression in Goldfish

Seven day feeding of high carbohydrate and very high fat (20%) significantly reduced NUCB1 mRNA expression in the hypothalamus (F value – 13.92; P value – 0.017) (**Figure 2.4 a**). No changes were elicited by high protein and 7% fat diet (F value – 2.57; P value – 0.56). Goldfish fed on high carbohydrate, high fat (9%) and very high fat diet (20%) for 28 days had significant attenuation in NUCB1 mRNA expression in the hypothalamus (P value – 0.94) (**Figure 2.4 b**). Similar to 7-day results, protein feeding did not cause any changes in NUCB1 mRNA expression in the hypothalamus (F value – 1.3; P value – 0.71) (**Figure 2.4 b**). High carbohydrate and high fat (9% and 20%) feeding for 7 days downregulated NUCB1 mRNA expression in the gut (F value – 24.34; P value – 0.44) (**Figure 2.4 c**). High protein diet feeding for 7 days did not elicit any changes in gut (P value – 0.23), but decreased NUCB1 expression at 28 days (; P value – 0.011) (**Figure 2.4 c, d**). Both high carbohydrate and very high fat also caused a similar decrease in NUCB1 mRNA in the gut after 28 days of feeding (P value – 0.032) (**Figure 2.4 d**).

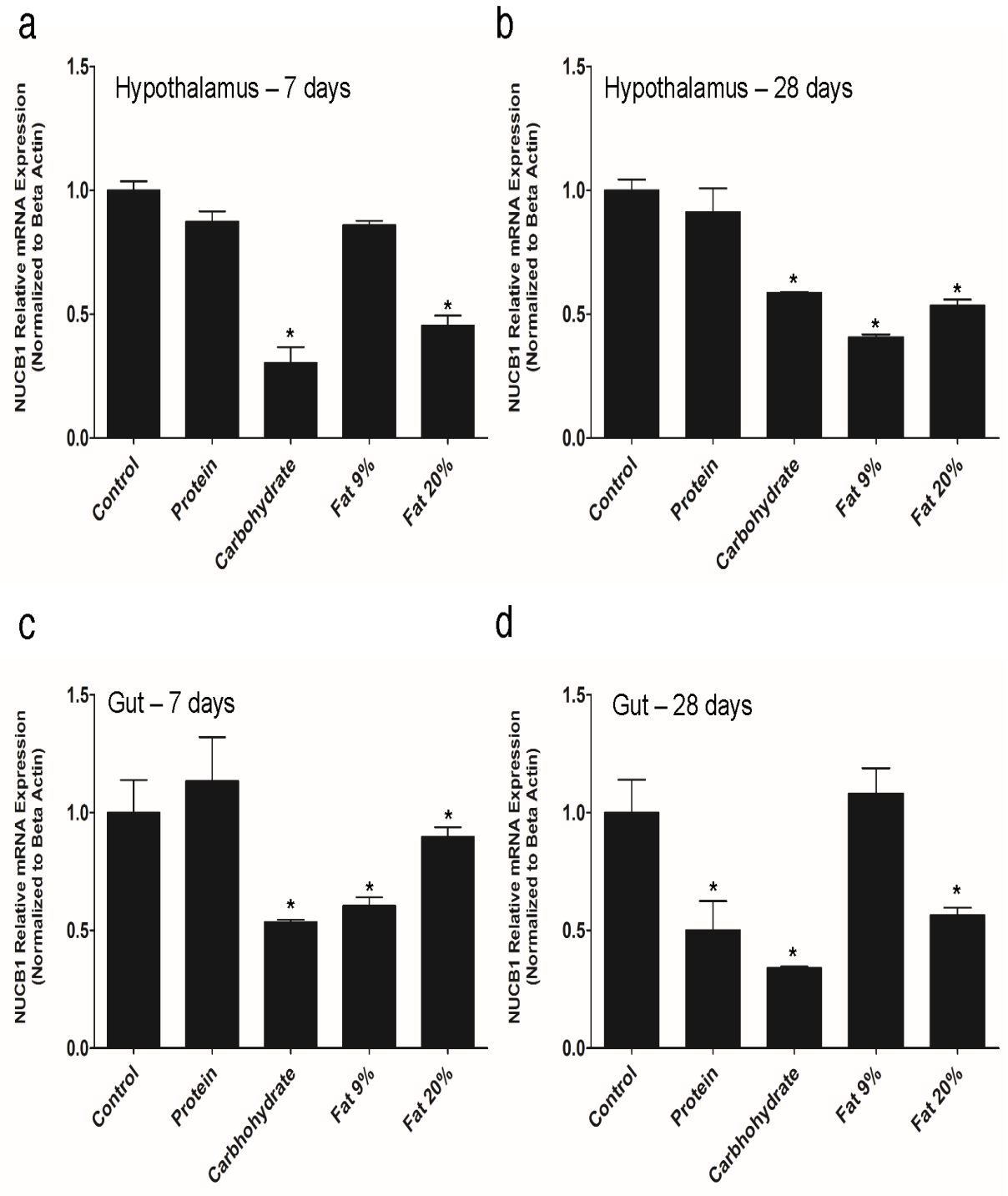

a
Hypothalamus – 7 days
NUCB1 Relative mRNA Expression
(Normalized to Beta Actin)
1.5
1.0
0.5
0.0
*
*
*
Control
Protein
Carbohydrate
Fat 9%
Fat 20%
b
Hypothalamus – 28 days
NUCB1 Relative mRNA Expression
(Normalized to Beta Actin)
1.5
1.0
0.5
0.0
*
*
*
Control
Protein
Carbohydrate
Fat 9%
Fat 20%
c
Gut – 7 days
NUCB1 Relative mRNA Expression
(Normalized to Beta Actin)
1.5
1.0
0.5
0.0
*
*
*
Control
Protein
Carbhohydrate
Fat 9%
Fat 20%
d
Gut – 28 days
NUCB1 Relative mRNA Expression
(Normalized to Beta Actin)
1.5
1.0
0.5
0.0
*
*
*
Control
Protein
Carbhohydrate
Fat 9%
Fat 20%

Figure 2.4. NUCB1 mRNA expression in goldfish hypothalamus after 7 days or 28 days of feeding special diets containing varying amounts of macronutrients. Groups include different diets tested: Control, Protein, Carbohydrate, Fat (9%); Fat (20%). Results are mean + SEM. Asterisks denote significant differences ($P < 0.05$) between the various diets compared to control.

2.3.5. NUCB1 mRNA Expression in Gut and Pituitary Follows a Circadian Pattern

Goldfish maintained under a 12L:12D photoperiod and scheduled feeding at ZT-4 exhibited a rhythmical expression of NUCB1 mRNAs in the gut, with a significant increase during the dark phase of the cycle (ZT-16) that remained until feeding time (F value – 28.23; P value – 0.0448) (**Figure 2.5 a**). NUCB1 mRNA expression was observed to be rhythmic also in the pituitary, exhibiting a daily expression profile characterized by one peak during the light and another during the dark phase of the circadian cycle (F value – 8.60; P value – 0.0112) (**Figure 2.5 e**). No daily rhythms in NUCB1 mRNA expression were detected in forebrain (F value – 0.05; P value – 1.34) (**Figure 2.5 b**), hindbrain (F value – 2.3; P value – 0.44) (**Figure 2.5 c**) and hypothalamus (F value – 9.7; P value – 0.43) (**Figure 2.5 d**).

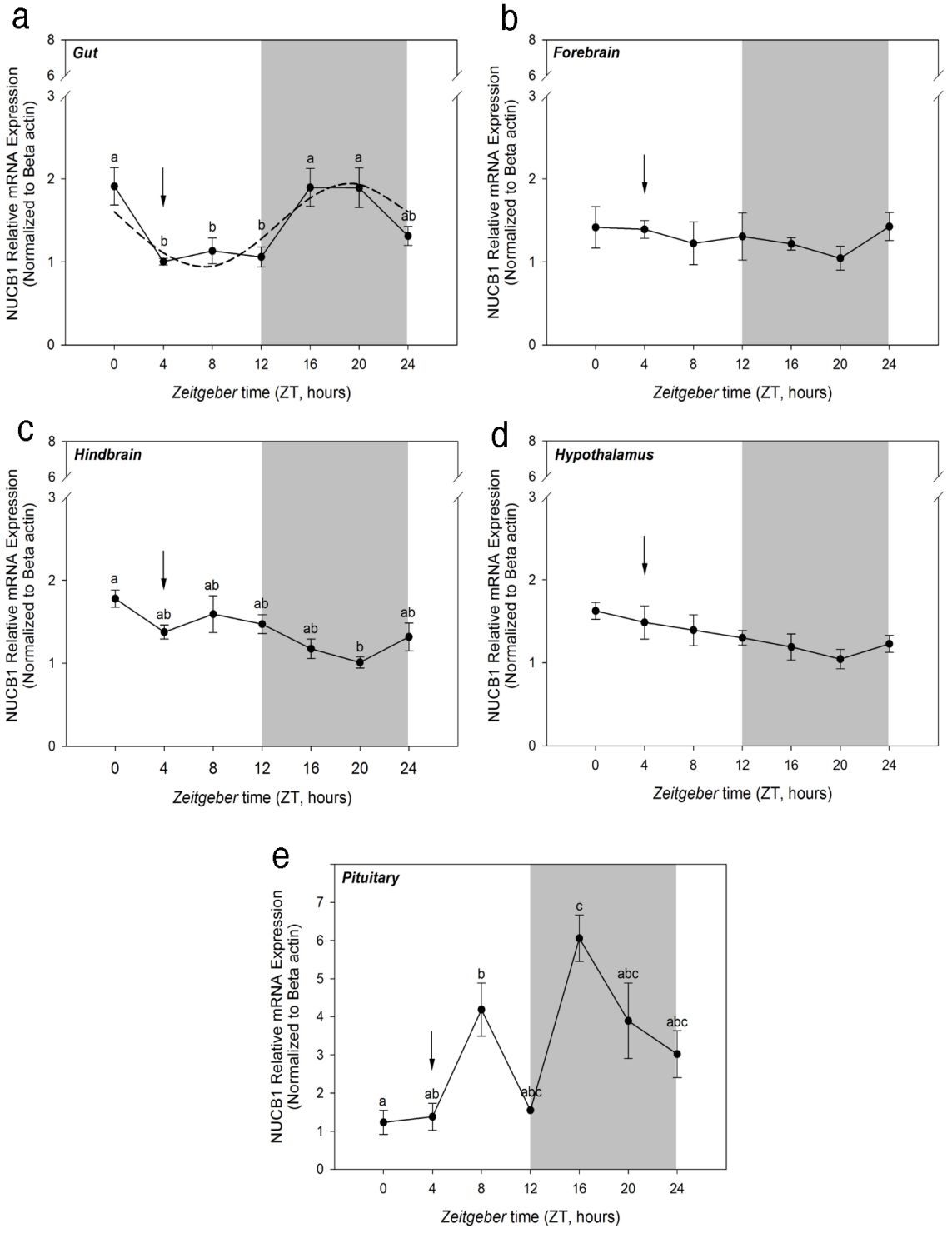

a
Gut
NUCB1 Relative mRNA Expression
(Normalized to Beta actin)
Zeitgeber time (ZT, hours)
b
Forebrain
c
Hindbrain
d
Hypothalamus
e
Pituitary

Figure 2.5. NUCB1 mRNA expression displayed a rhythmic pattern in the gut and pituitary of goldfish. Expression of NUCB1 mRNA expression in the gut **(a)**, forebrain **(b)**, hindbrain **(c)**, hypothalamus **(d)** and pituitary **(e)** of goldfish maintained under 12L: 12D photoperiod and scheduled feeding. The mRNA expression data was normalized to beta-actin. Data are presented as mean + SEM. The grey areas represent the night time and arrows indicate feeding time. Different alphabets denote significant differences between the time points ($P<0.05$, n = 6 fish/group). One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. The dashed line in figure a indicates a significant rhythm determined by cosinor analysis.

2.3.6. Testosterone and Estradiol Modulate NUCB1 mRNA in Goldfish

NUCB1 mRNA expression was downregulated in pituitary of goldfish that received estradiol (100 µg/g body weight) (P value – 0.02) (**Figure 2.6 a**). NUCB1 mRNA expression was upregulated in gut, hindbrain and hypothalamus of testosterone (100 µg/g body weight) treated goldfish (**Figure 2.6 b**) (*P value – 0.0226; **P value – 0.0078; ***P value – 0.0004). Estradiol and testosterone caused no other effect on NUCB1 mRNA expression in other tissues (P value – 0.(**Figures 2.6 a-b**).

2.3.7 Food Deprivation Suppresses NUCB1 mRNA Expression

A significant decrease in NUCB1 mRNA expression was observed in hypothalamus, midbrain, pituitary and hindbrain in response to food deprivation for 3 days (F value – 16.4; P value – 0.045) (**Figure 2.6 c**). No change in gut NUCB1 mRNA was detected after 3 days of food deprivation (P value – 0.02). NUCB1 expression was significantly decreased in both brain and gut after 7 days of food deprivation (F value – 24.34; P value – 0.031) (**Figure 2.6 d**).

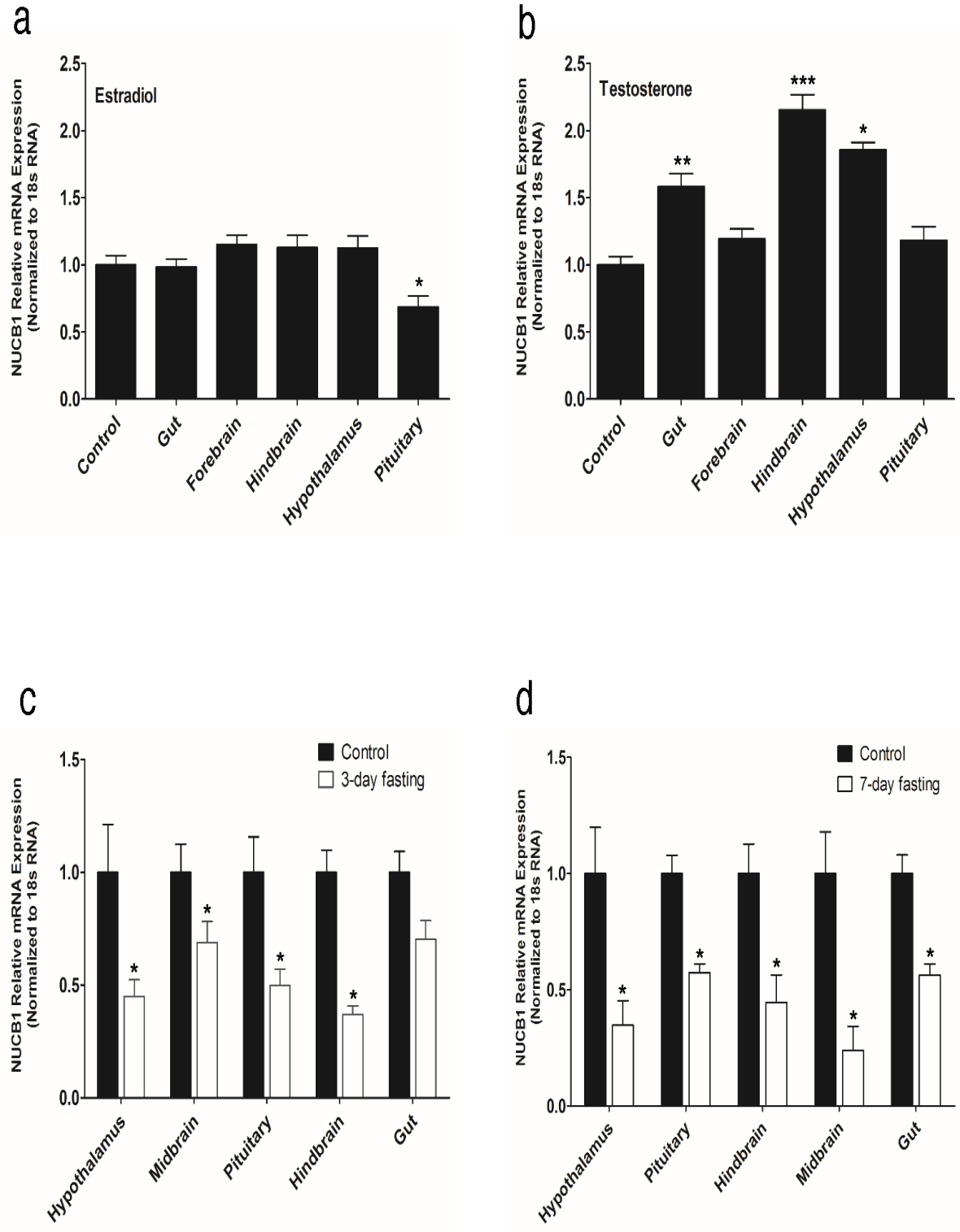
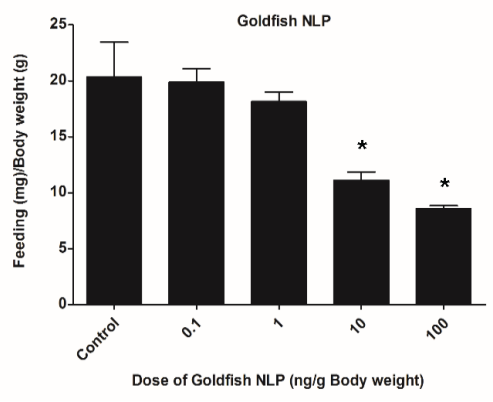

a
Estradiol
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Gut
Forebrain
Hindbrain
Hypothalamus
Pituitary
b
Testosterone
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
**
***
*
Control
Gut
Forebrain
Hindbrain
Hypothalamus
Pituitary
c
Control
3-day fasting
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
1.5
1.0
0.5
0.0
Hypothalamus
Midbrain
Pituitary
Hindbrain
Gut
d
Control
7-day fasting
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
1.5
1.0
0.5
0.0
Hypothalamus
Pituitary
Hindbrain
Midbrain
Gut

Figure 2.6. Estradiol (100 µg/g B.W) decreased NUCB1 mRNA expression in goldfish pituitary and food deprivation suppressed NUCB1 mRNA expression in goldfish. Expression of NUCB1 in gut, forebrain, hindbrain, hypothalamus and pituitary of fish treated with estradiol **(a)** or testosterone **(b)** were normalized to control group. The mRNA expression data were normalized to 18s RNA. Asterisks denote significant differences between the control and treatment tissues in goldfish (* $p<0.05$, ** $p<0.01$, *** $p<0.005$, n = 6 fish/group). The mRNA expression data were normalized to 18s RNA. Asterisks denote significant differences between control and fasted groups (* $p<0.05$, n = 6 fish/group). Data is represented as mean + SEM. Student-Newman-Keuls test **(a, b)** and One-way ANOVA followed by Tukey's multiple comparison test (**c, d**) were used for statistical analysis.

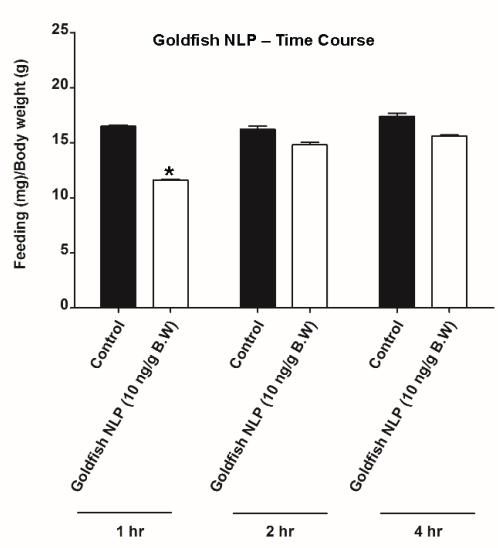
2.3.8. NLP Reduces Food Intake in Goldfish

Administration of 10 and 100 ng/g body weight goldfish NLP (**Figure 2.7 a**) and rat NLP (**Figure 2.6 c**) reduced food intake in goldfish at 1-hour post-injection (F value – 15.82; P value – 0.038). No significant changes in food intake were found in goldfish NLP injected fish at 2 and 4 hours post-injection (P value – 0.13) (**Figure 2.7 b**). No effect on food intake was observed in response to an intraperitoneal injection of 100 ng/g body weight scrambled peptide (P value – 1.23) (**Figure 2.7 d**), while rat NLP significantly reduced food intake (P value – 0.78) (**Figure 2.7 d**).

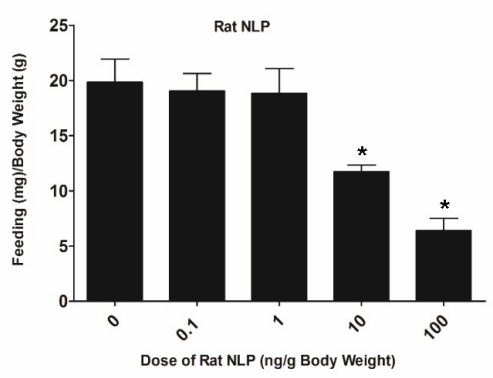
a


Goldfish NLP
Feeding (mg)/Body weight (g)
25
20
15
10
5
0
*
*
Control
0.1
1
10
100
Dose of Goldfish NLP (ng/g Body weight)

b


Goldfish NLP – Time Course
Feeding (mg)/Body weight (g)
25
20
15
10
5
0
*
Control
Goldfish NLP (10 ng/g B.W)
Control
Goldfish NLP (10 ng/g B.W)
Control
Goldfish NLP (10 ng/g B.W)
1 hr
2 hr
4 hr

c


Rat NLP
Feeding (mg)/Body Weight (g)
25
20
15
10
5
0
*
*
0
0.1
1
10
100
Dose of Rat NLP (ng/g Body Weight)

d

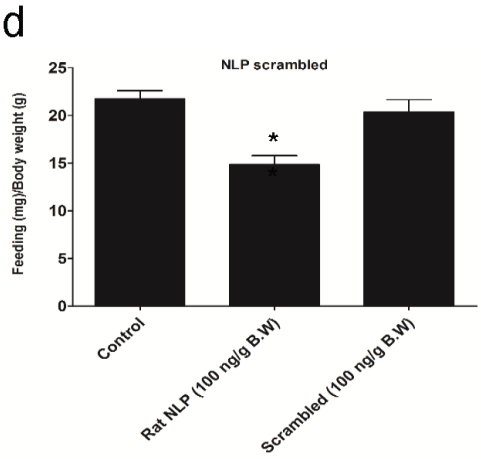

NLP scrambled
Feeding (mg)/Body weight (g)
25
20
15
10
5
0
*
Control
Rat NLP (100 ng/g B.W)
Scrambled (100 ng/g B.W)

Figure 2.7. NLP reduced food intake in goldfish. Groups include: Control (0); Dose of Goldfish NLP (0.1, 1, 10, 100); Dose of Rat NLP (0.1, 1, 10, 100); Scrambled (100 ng/g B.W). Asterisks denote significant differences between control and NLP injected groups of the same ($*p < 0.05$, $n = 10$ fish/group). Data are represented as mean + SEM. T-test (b) and One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

2.3.9. NLP Downregulates Preproghrelin and Orexin-A mRNA Expression and Upregulates CART mRNA Expression

Goldfish NLP (1, 10, 100 ng/g B.W) downregulated preproghrelin (**Figures 2.8 a,d**) and orexin-A (**Figures 2.8 b,e**) mRNA expression in hypothalamus and gut of goldfish (F value – 15.54; P value – 0.03). Goldfish NLP (100 ng/g B.W) also upregulated CART mRNA expression in the hypothalamus (F value – 20.46; P value – 0.045) (**Figure 2.8 c**) and gut (F value – 23.3; P value – 0.026) (**Figure 2.8 f**) of goldfish. Synthetic rat NLP (10 and 100 ng/g body weight) downregulated preproghrelin (**Figures 2.8 g, 2.9 a**) and orexin-A (**Figures 2.8 h, 2.9 b**) mRNA expression in the hypothalamus (P value – 0.017) and gut (P value – 0.033). Rat NLP had no effects on CART mRNA expression (P value – 0.19) (**Figures 2.8 i, 2.9 c**). No effect for the scrambled peptide was found on preproghrelin (P value – 0.77), orexin-A (P value – 0.10) and CART mRNA expression (P value – 0.98) (**Figures 2.9 d-i**).

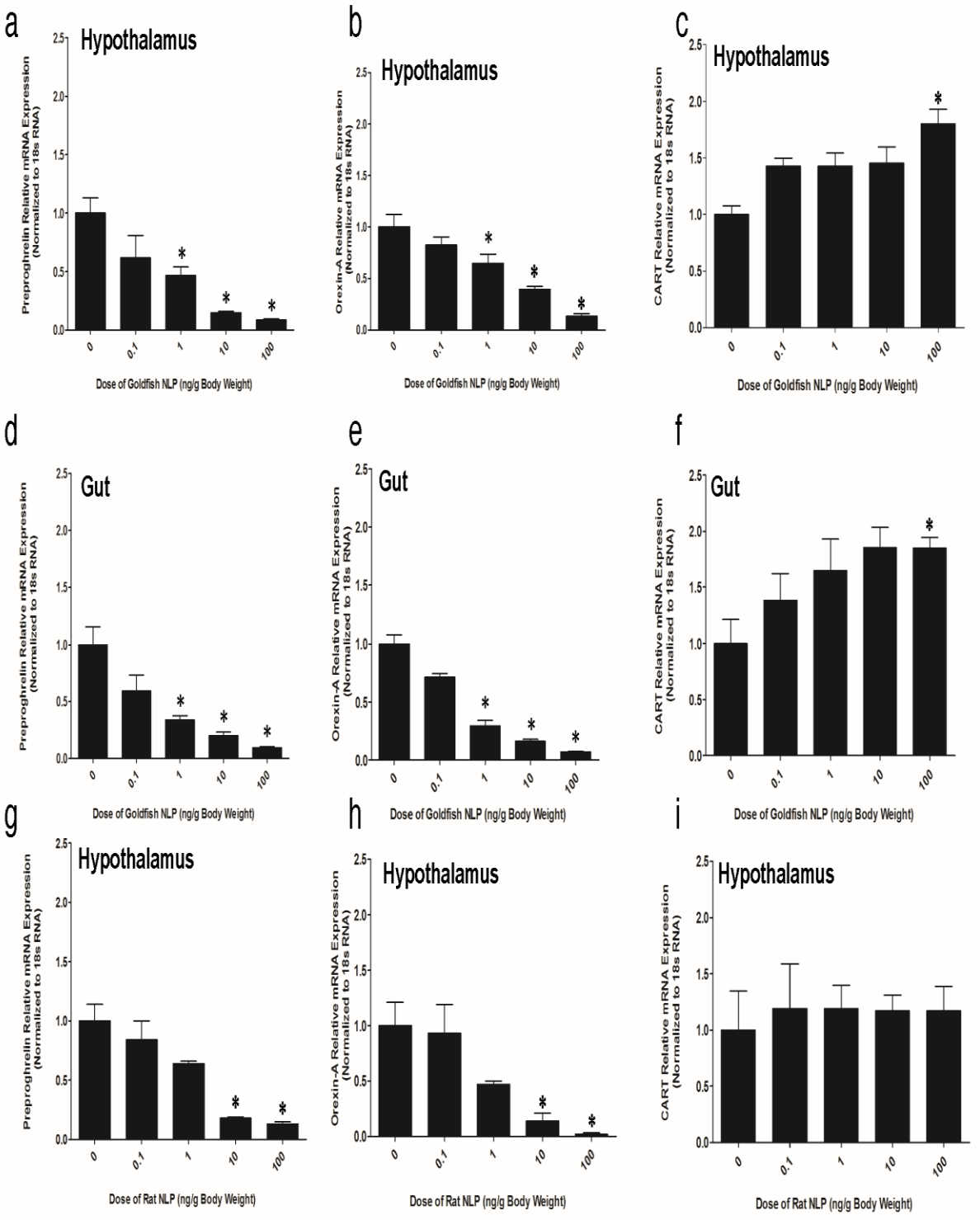

a
Hypothalamus
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
b
Hypothalamus
Orexin-A Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
c
Hypothalamus
CART Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
d
Gut
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
e
Gut
Orexin-A Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
f
Gut
CART Relative mRNA Expression (Normalized to 18s RNA)
Dose of Goldfish NLP (ng/g Body Weight)
g
Hypothalamus
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)
h
Hypothalamus
Orexin-A Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)
i
Hypothalamus
CART Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)

Figure 2.8. NLP downregulates appetite regulatory proteins in goldfish. Intraperitoneal injection of goldfish and rat NLP on appetite regulatory proteins in goldfish. Groups include: Control (0); Dose of Goldfish NLP (0.1, 1, 10, 100); Dose of Rat NLP (0.1, 1, 10, 100); Scrambled (100 ng/g B.W). The mRNA expression data was normalized to 18 s RNA. Asterisks denote significant differences between control and NLP injected groups ($*p < 0.05$, $n = 10$ fish/group). Data are presented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis.

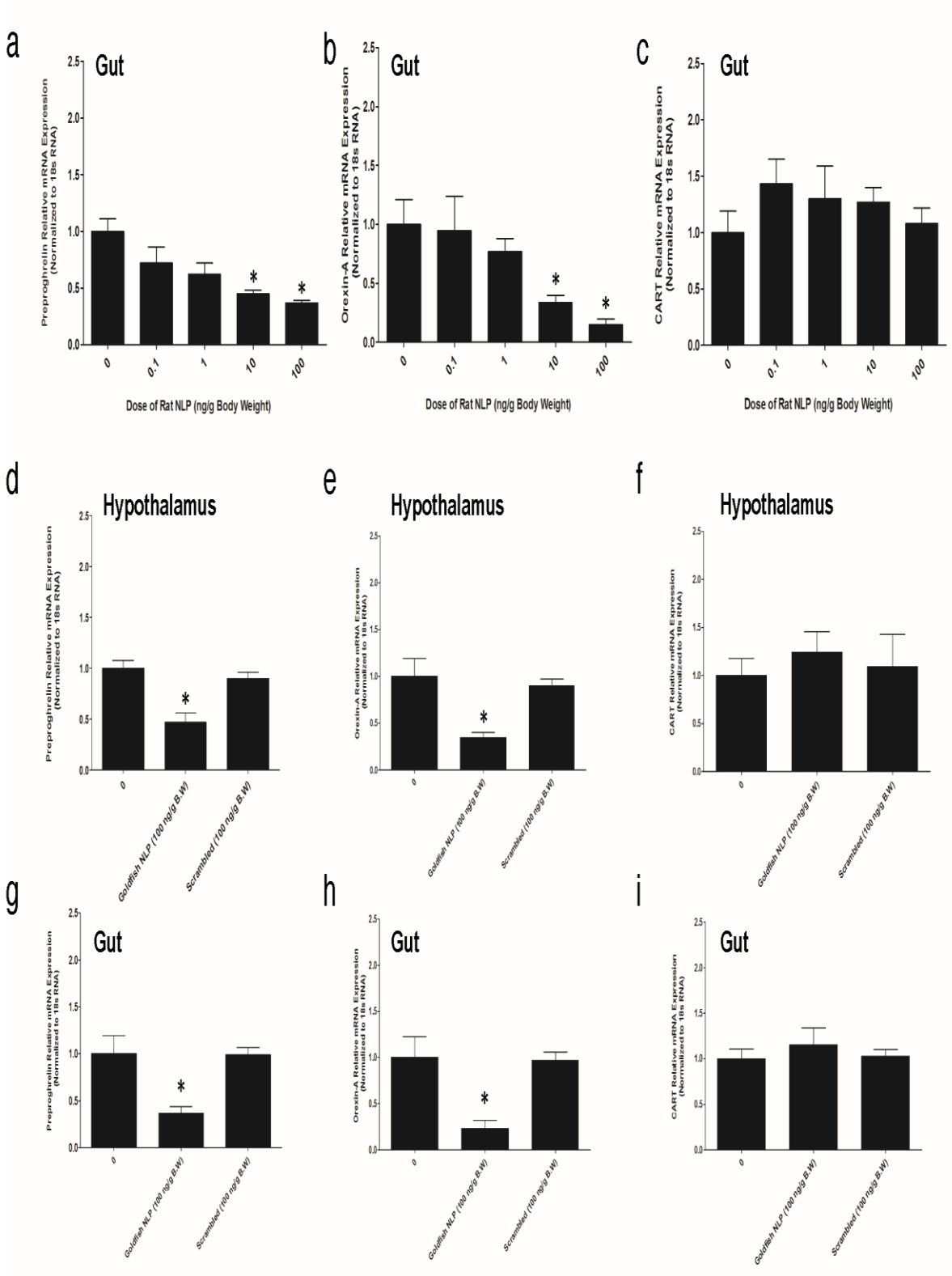

a
Gut
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)
b
Gut
Orexin-A Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)
c
Gut
CART Relative mRNA Expression (Normalized to 18s RNA)
Dose of Rat NLP (ng/g Body Weight)
d
Hypothalamus
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
Goldfish NLP (100 ng/g B.W)
Scrambled (100 ng/g B.W)
e
Hypothalamus
Orexin-A Relative mRNA Expression (Normalized to 18s RNA)
f
Hypothalamus
CART Relative mRNA Expression (Normalized to 18s RNA)
g
Gut
Preproghrelin Relative mRNA Expression (Normalized to 18s RNA)
h
Gut
OrexinA Relative mRNA Expression (Normalized to 18s RNA)
i
Gut
CART Relative mRNA Expression (Normalized to 18s RNA)

Figure 2.9. Rat NLP downregulated preproghrelin and orexin-mRNA expression in gut of goldfish. Intraperitoneal injection of rat NLP on appetite regulatory proteins in goldfish. Groups include: Control (0); Dose of Rat NLP (0.1, 1, 10, 100); Scrambled (100 ng/g B.W). The mRNA expression data was normalized to 18 s RNA. Asterisks denote significant differences ($*p < 0.05$) between control and NLP injected groups. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Data are presented as mean + SEM. $n = 6–10$ fish/group.

2.4. Discussion

We recently discovered that NUCB1 encoded NLP is insulinotropic in mice [227]. Our *in silico* analysis of NUCB1 sequences from different species found that NLP is very highly conserved among vertebrates. Here, we report the discovery of anorectic actions of NLP in fish, its tissue distribution and regulation of endogenous NUCB1. NLP sequence is conserved in zebrafish and share high sequence identity (74%) with the M30 region of zebrafish NUCB2a and NUCB2b [282]. Previous research has shown that the M30 region of nesfatin-1 is critical for the satiety effects of nesfatin-1 [278]. Considering the high sequence identity between the M30 region of nesfatin-1 and the corresponding region in NLP, it is highly likely that NLP has anorexigenic actions similar to nesfatin-1. NUCB1/NLP expression was abundant in the hypothalamus, hindbrain and midbrain while comparatively low expression was detected in other tissues. The results presented here are the first line of evidence for NUCB1 expression in the central nervous system. These results are in line with previous studies that showed abundant NUCB2 expression in appetite regulatory centers of hypothalamus in rats [32] and goldfish [95, 281]. Vast distribution of NUCB1 in fish tissues explains that NUCB1 might have central and peripheral effects. Our results indicate that hypothalamus is an abundant source of NUCB1 in goldfish suggesting a role for NLP in the central control of reproduction and metabolism.

To gain further understanding on the cell specific expression of NUCB1/NLP, we conducted immunofluorescence studies. NUCB1/NLP-like immunoreactivity was observed in the pars distalis region of goldfish anterior pituitary. NUCB1/NLP-like immunoreactivity was observed in the rostral pars distalis (RPD) region of goldfish anterior pituitary. RPD cells in goldfish are generally melanotrophs [281]. These observations are in agreement with previous

results that indicated NUCB2/nesfatin-1-like immunoreactivity in the rostral pars distalis (RPD) and pars intermedia (PI) in goldfish pituitary [281]. NUCB2/nesfatin-1 is also present in mice pituitary [288]. The presence of NUCB1/NLP-like ir in goldfish pituitary suggests a role for NUCB1 on pituitary hormone secretion. For example, nesfatin-1 regulates luteinizing hormone secretion in goldfish [281]. NUCB1/NLP-like ir was also detected in the mucosal cells lining the anterior intestine (J-loop). Previous studies have shown that NUCB2/nesfatin-1-like ir is found in the villi of goldfish gut and that nesfatin-1 suppresses ghrelin [280]. NUCB2/Nesfatin-1 like immunoreactivity was abundant in the glandular cells of gastrointestinal tract in rats [289], and glucose stimulates its release [290]. It is likely that NUCB1 and NLP are also secreted in a meal responsive manner from the gut. The current study also demonstrated the localization of NUCB1/NLP in goldfish reproductive organs. NUCB1/NLP-ir was observed in the ovarian thecal or follicular cells and in the Leydig cells of goldfish testis, and was found colocalized with Leydig cell marker SOX9 [286] and follicular and/or thecal cell marker FOXL2 [285]. The results are in line with previous findings of NUCB2/nesfatin-1-ir within the follicular cells of goldfish and zebrafish ovaries [281]. Previous reports have showed the localization of NUCB2/Nesfatin-1-ir in Leydig cells in Japanese quail [291] and rats [292]. The localization of NUCB1/NLP-ir in the ovary and testis suggests a possible role for NLP in gonadal physiology and sex steroid hormone production. The extensive cell specific localization of NUCB1/NLP in goldfish suggests multiple biological functions for NLP in fish.

Next, we determined some factors that regulate the tissue specific expression of NUCB1 mRNA. While it was found that macronutrients fat, carbohydrate and protein suppress NUCB1 mRNA expression, these effects were highly tissue specific and were dependent on the duration

of feeding. Although nutrient regulation of Nucleobindins in fish is understudied, the results reported here are in agreement with previous research in mice, where high fat and protein diets elicited a reduction in NUCB2 mRNA [33]. This meal responsive inhibition of NUCB1 mRNA is suggestive of a role for NUCB1/NLP in fish feeding. It was also detected that NUCB1 mRNA expression displayed a circadian pattern of expression. NUCB1 mRNA in goldfish pituitary peaked during the light (ZT-8) and dark phases (ZT-16). In the gut, NUCB1 mRNA expression was lowest during the light phase when the fish feeding occurs, and relatively higher in the dark phase. These are the first results on a circadian pattern of NUCB1/NLP expression in both central and peripheral tissues of a vertebrate. Estradiol treatment downregulated NUCB1 mRNA expression in goldfish pituitary, while testosterone upregulated NUCB1 expression in goldfish gut, hindbrain and hypothalamus. Chung et al [288] observed a significant increase in NUCB2 mRNA expression post estradiol administration in ovariectomized mice. The differences between the studies might be due to the use of ovariectomized mice, and/or species-specific differences in the interactions of gonadal steroids and NUCB1. Food deprivation is known to play a significant role in altering neuroendocrine factors that play critical role in reproduction and energy intake in several species [282, 293]. In goldfish, fasting for 7 or 28 days did not result in any significant changes in body weight and this result is in agreement with previous reports in goldfish [77, 294, 295]. NUCB1 expression in gut was significantly lower when fish were food deprived for one week [282]. This is consistent with previous study on NUCB2 expression in goldfish gut and Ya fish that showed a significant downregulation after 7-day food deprivation[95, 96]. Similar results were also reported in rodents, showing a decrease in NUCB2 expression in gastric endocrine cells [289]. The negative modulation of NUCB1 in response to fasting suggests anorexigenic actions for NUCB1 and/or encoded NLP. These new findings on

endogenous NUCB1, especially the energy status and circadian pattern dependence of its expression provide new insights on NUCB1/NLP biology in fish.

Our next study determined if NLP is indeed biologically active in goldfish. When comparing the mid segment (M30) region of zebrafish NLP and corresponding synthetic rat NLP region, it was found that both sequences have 80% amino acid sequence identity. Due to this very high sequence identity, we first injected goldfish with rat NLP. Intraperitoneal injection of synthetic rat NLP inhibited feed intake in goldfish by 40% and 68% at 10 and 100 ng/g body weight doses respectively over a period of 1 hour. Intraperitoneal injection of zebrafish NLP also decreased feed intake by approximately 30% and 60% corresponding to similar doses over a period of 1 hour in goldfish. To confirm that the NLP elicited satiety effect requires a specific sequence, we injected goldfish with a scrambled peptide based on NLP. The scrambled peptide did not elicit any changes in food intake of goldfish. These data clearly indicate an anorexigenic action for NLP in goldfish. Since NLP had a profound anorexigenic action in goldfish, we explored whether NLP influences other appetite regulatory factors, including ghrelin, orexin-A and cocaine and amphetamine regulated transcript (CART) to elicit its satiety effects.

Ghrelin and orexin-A are potent orexigens in goldfish [296, 297], and CART, is an anorexigen [298]. Nesfatin-1 was found to suppress ghrelin and orexin-A in goldfish [280]. Similar to this, a significant downregulation of ghrelin and orexin-A mRNA expression by NLP (10 and 100 ng/g B.W) was observed in goldfish hypothalamus and gut. In contrast, NLP administration (100 ng/g body weight) upregulated CART mRNA expression in goldfish gut. IP injected NLP could possibly cross the blood brain barrier in a manner similar to nesfatin-1 [299,

300] to induce satiety. These results show that NLP, similar to nesfatin-1 could suppress hormones in the orexigenic pathways and stimulate anorexigenic pathways to decrease food intake.

This research discovered several key aspects of NUCB1/NLP biology. First, it uncovered the satiety effects of NLP. Second, tissue and cell specific expression of NUCB1 was determined. Third, we found macronutrients, energy status, sex steroids and circadian period as four regulators of endogenous NUCB1. These results are of importance, and add significant new information to our growing knowledge on naturally occurring regulators of metabolic and endocrine functions in vertebrates. Our discoveries outlined here provide the first line of evidence on biological activity of NUCB1 encoded NLP in goldfish. The processing of endogenous NLP from NUCB1 and its mode of action are important new directions to consider during future investigations on Nucleobindins and NLP.

TRANSITION

The following chapter focuses on the final objective of my thesis: Nesfatin-1-Like Peptide (NLP) regulates cardiovascular physiology in zebrafish. Previous results from our lab have shown nesfatin-1 effects on cardiovascular physiology in zebrafish. Since NUCB1 has more similarity with NUCB2 and nesfatin-1, we investigate whether NLP/NUCB1 also has some effect on cardiovascular functions. We conducted an ultrasound study to determine the role of endogenous NLP in modulating cardiac functions. This part of my thesis was conducted in collaboration with Dr. Lynn Weber.

Publication: Sundarrajan L, Jithine JR, Weber LP, Unniappan S., 2018. Nesfatin-1-Like Peptide (NLP) Modulates Cardiovascular Functions and Affects Calcium Handling Proteins in Zebrafish. *Manuscript in Preparation*.

Contributions: The project was in collaboration with Dr. Lynn Weber and shared the ultrasound equipment and assisted us during the ultrasound studies in zebrafish. Sundarrajan planned and executed all and prepared the manuscript draft. Jithine helped in conducting ultrasound studies and tissue collection. Dr. Suraj Unniappan provided the original ideas, funding for this research. Dr. Lynn Weber and Dr. Suraj Unniappan helped design experiments, assisted with *in vivo* studies, tissue sampling, data analysis and interpretation and manuscript preparation.

Chapter 3

Nesfatin-1-Like Peptide (NLP) Modulates Cardiovascular Functions and Affects Calcium Handling Protein Encoding mRNAs in Zebrafish

3.1. Introduction

Nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-Influencing proteiN-1) is an 82-amino acid anorexigenic peptide encoded in the N-terminal region of nucleobindin-2 (NUCB2) [32]. NUCB2 is cleaved by prohormone convertases (PC 1/3 and 2) resulting in three peptide fragments, of which nesfatin-1 known to be biologically active [32]. NUCB2 was named so due to its high sequence similarity with another secreted protein, NUCB1. Both NUCB2 and NUCB1 belong to a class of multi-domain Ca^{2+} and DNA binding proteins that play an important role in cell signaling [220]. NUCB1 and NUCB2 exhibit 62% amino acid identity within their bioactive regions (24-53 amino acids; mid segment M-30) between humans and zebrafish [221-223]. Administration of the bioactive core (M30, mid-segment) of nesfatin-1 inhibits food intake in rodents, pigs and fish [278, 301] and body mass in rodents. In mice, nesfatin-1 secretion is modulated by nutrients suggesting that nesfatin-1 plays an important role in metabolism and energy homeostasis [33]. Administration of nesfatin-1 (25 pmol/rat) affected thermogenesis and resulted in energy expenditure and a reduction in food intake of rats [271]. Similarly, nesfatin-1 administration inhibited food intake and reproductive hormone secretion [95, 280, 281] in goldfish. Besides its role in feeding and metabolism, nesfatin-1 is known to regulate cardiac

functions [20, 21, 302, 303]. Administration of nesfatin-1 protected the myocardial tissue in rats treated with isoproterenol [194]. Central injections of nesfatin-1 increased plasma catecholamine levels, stimulated vasopressin and renin and enhanced sympathetic activity [195]. In zebrafish, nesfatin-1 immunoreactivity (IR) was detected in cardiomyocytes, and intraperitoneal administration of nesfatin-1 reduced cardiovascular functions and affected calcium handling proteins [21].

Our lab reported the discovery of a nesfatin-1-like peptide (NLP) in mice and its insulinotropic actions on mice pancreatic beta cells [227]. In male Wistar rats, administration of NLP at 100 μg/kg B.W reduced food intake and played an important role in modulating whole body energy balance [16]. In goldfish, NLP (10, 100 ng/g B.W) reduced food intake and affected appetite regulatory proteins [15]. More recently, NUCB1 gained attention due to its similarity with NUCB2 and nesfatin-1. For example, our *in silico* analysis found that NUCB1 in fish and mammals encode a nesfatin-1 like sequence [220, 227], and these peptides possess prohormone convertase sites that enable its processing [227]. Immunofluorescence studies also revealed that the localization of NUCB1 is highly concentrated in the pancreatic islets of mice [227], and colocalizes insulin [304]. NUCB1 is very highly conserved in mammals and non-mammals [15, 220, 222, 227]. NUCB1 has been shown to play an important role in maintaining Ca2+ homeostasis and it interacts with G proteins and cyclooxygenases [38, 305]. Is NUCB1/NLP that exhibits structural similarities to NUCB2/nesfatin-1, and shares common functions as a regulator of cardiac physiology in zebrafish? We hypothesize that NLP modulates cardiovascular functions and alters the expression of mRNAs encoding cardiac proteins in zebrafish. Using zebrafish as a model organism and employing the ultrasound bio-microscopy (UBM), we

conducted *in vivo* studies to test this hypothesis. Our current study aimed to determine if NLP modulates cardiovascular function, and affected calcium-binding proteins in fish. First, we elucidated whether NLP has any whole-body effects, by examining cardiovascular function in zebrafish and goldfish. Second, we determined whether NLP affected irisin, a cardio-regulator and skeletal muscle protein in zebrafish. Finally, we studied NLP effects on the expression of calcium binding proteins, ATP2a2a and RyR1b in zebrafish. NLP downregulated cardiac functions in zebrafish, and decreased the expression of irisin (a cardiac regulator), and calcium handling RyR1b mRNAs in zebrafish. In contrast, another calcium handling protein ATP2a2a encoding mRNA in cardiomyocytes was found elevated in response to NLP.

3.2. Materials and Methods

3.2.1. Animals

Male and female zebrafish (*Danio rerio*; 3-4 months old; body weight ~1.5 g) and goldfish (*Carassius auratus*, common variety, 4-5 inches long, body weight ~ 25 g, common variety) were purchased from Dalhousie University (Canada) or from a local supplier arranged by the University of Saskatchewan animal care office. Zebrafish were maintained at 27 °C and goldfish at 24 °C under a 12L:12 D photoperiod cycle. Fish were group housed (n=6 fish/tank) and fed once a day (10:00 AM everyday) with 4% body weight slow sinking pellets (Aqueon, Catalog # 06053). For tissue collection, fish were anesthetized in 0.5% TMS-222 followed by spinal transection. The animal studies performed were compiled within the policies of the Canadian Council for Animal Care and approved by University of Saskatchewan Animal Research Ethics Board (2012-0033).

3.2.2. Immunohistochemical Localization of NUCB1

The localization of the NUCB1 in zebrafish heart and skeletal muscle tissue sections were detected by immunohistochemical (IHC) studies as described in detail earlier (**Chapter 2.2**). The primary antibody used was a custom synthesized rabbit polyclonal anti-mouse nucleobindin-1 (1:3000, Pacific Immunology, Ramona, CA) for NUCB1. The slides were then washed with PBS and then were incubated with secondary antibody for one hour at room temperature. Goat polyclonal anti-rabbit IgG (Catalog # T1-1000, 1:500, Vector Laboratories, California) was used as secondary antibody for NUCB1 respectively. The slides were then rewashed with PBS and water and were mounted on Vectashield medium containing DAPI dye

(Blue, Vector Laboratories). The slides were dried and imaged using a Nikon inverted microscope (L100) (Nikon DS-Qi1 MC camera, ON, Canada). The images were analysed using NiS Elements imaging software (Nikon, Canada). For negative controls, slides were incubated either with secondary antibody alone (no primary antibody control), or preabsorption using synthetic goldfish NLP. Since the antibody used here detects both NUCB1 and NLP, we used NUCB1/NLP-like immunoreactivity to refer to the staining obtained in our IHC studies.

3.2.3. Dose Dependent Effects of NLP on Cardiac Function in Zebrafish and Goldfish

Cardiovascular function was assessed in zebrafish using a VEVO 3100 high frequency ultrasound machine (Visualsonics, Markham, ON) using B-mode imaging as described earlier [21, 122]. Zebrafish were injected intraperitoneally (6 μL) with 10 ng/g B.W) synthetic rat NLP (VPVDRAAPHQEDNQATETPDTGLYYHRYLQEVINVLETDGHFREKLQAANAEDIKSGL SQELDFVSHNVRTKLDEL, Abgent Technologies, California, >95% purity), or goldfish/zebrafish NLP (VPIDRNPDPPQEEKAEENVDTGLYYDRYLREVIEVLETDPHFREK LQTANTEDIKNGRLSKELDLVGHHVRTRLDEL, Pacific Immunology, Ramona, CA, >95% purity) at four different doses (0.1, 1, 10, 100 ng/g B.W). For control, zebrafish was injected with 6 μL of saline (0.9 % sodium chloride, Baxter Corporation, Catalog # JB1323). Scrambled NLP was designed to test whether a peptide that shares the same amino acids and length of rat NLP, but was highly dissimilar in the arrangement could elicit biological effect on zebrafish. Scrambled peptide was designed using the Sequence Manipulation Suite™ online tool (www.bioinformatics.org/sms2/). NLP scrambled peptide (PDSRSDDGSPSVQLQDYALIADAEVTLTHIELFGSPQNATKLLNKTERLRFLKVVRGKH

RENVVATEHYQAQKYPEEDE) with the lowest similarity to the rat NLP sequence was selected. Zebrafish were injected intraperitoneally with scrambled NLP (10 ng/g B.W). Each zebrafish was allowed to recover for a period of 15 min. Zebrafish (n=6 fish/group) was anaesthetized prior to ultrasound experiments using 20 mg/L Aquacalm (Syndel Laboratories, Canada). Zebrafish was then transferred to a Styrofoam holding dish and maintained at 27±0.5 °C with recirculating water to maintain anesthesia containing Aquacalm (20 mg/L) throughout the ultrasound testing and minimal impact towards cardiovascular function in zebrafish [21, 122].

Similar studies were conducted in goldfish, another cyprinid [248]. Briefly, goldfish (n=6 fish/ group) were intraperitoneally injected (100 μL) with effective dose of NLP (10 ng/g B.W) from our earlier zebrafish experiments. Goldfish were also injected with rat NLP (10 ng/g B.W) and scrambled NLP (10 ng/g B.W) and the fish were allowed to recover for a period of 15 min. Goldfish (n=6 fish/group) were anesthetized using 20 mg/L Aquacalm (Syndel Laboratories, Canada) and were transferred to the holding dish. The holding dish was maintained at 24 ±0.5 °C with aeration and recirculating water containing Aquacalm (20 mg/L) to maintain the fish under anaesthetic conditions throughout ultrasound testing and causing minimal impact to cardiac function in goldfish [21, 122].

A MX700 scan head was used to obtain short and long axis of the zebrafish and goldfish ventricle in B-mode. Areas of three different short axis along the ventricle were measured as A1, A2 and A3 while the ventricular length of long axis view was measured and divided by three to give ventricular height (h) as per Eq (1). All of these values were measured at both systole and

diastole volumes using Visualsonics software (Markham, ON). Using these values, end systolic and diastolic volumes (mm^3 = μl) were calculated using the equation:

$$V= (A1+A2)\ h + ((A3h)/2) + (\pi/ 6\ (h^3) \quad (1)$$

Stroke volume (SV) was calculated by subtracting end diastolic volume from end systolic volume. Heart rate (bpm) was calculated by counting number of heart beats per 10s during B-mode ultrasound loop video. Cardiac output was measured by multiplying heart rate and stroke volume (SV):

$$\text{Cardiac output} = \text{bpm} * \text{SV} \quad (2)$$

The body weight of each fish was noted, and cardiac volume and output data were normalized to body weight and statistical analysis was conducted.

3.2.4. Effect of NLP on Irisin mRNA Expression in Zebrafish

It has been shown that irisin regulates cardiac function in zebrafish [306]. We wanted to elucidate whether administration of NLP (I.P) modulated irisin mRNA expression in zebrafish heart and skeletal muscle. After ultrasound monitoring of zebrafish, heart and skeletal muscle were collected from all groups post 1 hour injection of zebrafish/goldfish NLP (0, 0.1, 1, 10, 100 ng/g B.W), rat NLP (10 ng/g B.W) and scrambled NLP treated groups of zebrafish. Total RNA extraction, cDNA synthesis and RT-qPCR studies were performed as described earlier (**2.2.3**). We wanted to determine whether irisin affects NUCB1 mRNA expression in the heart. To achieve this, NUCB1 mRNA expression in the heart and skeletal muscle already collected from

irisin treated zebrafish (**Chapter 5.2**) were used. Target mRNA expression was normalized to 18s RNA (housekeeping gene) (**Table 3.1**).

Table 3.1. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Gene Bank Accession Number
	Forward	Reverse		
Irisin	GCTTATATCTTC GCGTCCTC	GCCAGTTTCTC TGACTCTTT	58	NM_001044337.1
NUCB1	CTGTCTCTGTGT CTGCTGGT	TGGTGCTGTCCAG TTTAGCC	60	KU903286.1
18s	GGATGCCCTTA ACTGGGTGT	CTAGCGGCGCAA TACGAATG	60	KY486501.1

3.2.5. Expression of RyR1b and ATP2a2a mRNA Expression in Zebrafish

Nesfatin-1 affects calcium handling protein, SERCA2a (gene encoding for ATP2a2a in zebrafish) [260]. We wanted to determine whether NLP elicits similar effects on cardiomyocytes. Post ultrasound monitoring, heart tissues were collected from all groups of zebrafish. Tissue sampling was done approximately 1-hour post NLP injection from control fish, and fish treated with goldfish/zebrafish NLP, rat NLP or scrambled NLP. Total RNA extraction, cDNA synthesis and RT-qPCR were performed as described earlier (**2.2.3**). The primer sequences of zebrafish SERCA2a and ATP2a2a are shown in **table 3.2,** and the mRNA expression was normalized to 18s RNA (housekeeping gene).

Table 3.2. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Reference / Gene Bank Accession Number
	Forward	Reverse		
ATP2a2a	ATTTACTTGTGCGGATTCTTCTA	CACGATGTCTTTGGCTTTGA	60	[21]
RyR1b	CCGCTCCTTTGGACCTCAAT	ATCCTCAACACCATGACCGC	62.7	[21]
18s	GGATGCCCTTAACTGGGTGT	CTAGCGGCGCAATACGAATG	60	KY486501.1

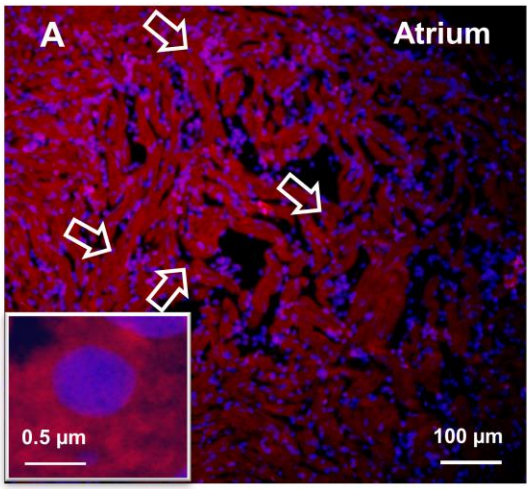
3.2.6. Statistical Analysis

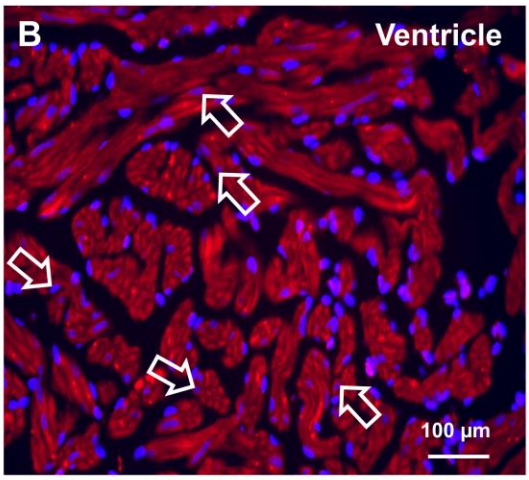
Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using PRISM version 5 (GraphPad Inc., USA) and IBM SPSS™ version 21 (IBM, USA) were used for statistical analysis. $*P < 0.05$ was considered statistically significant. Data are represented as mean + SEM. For ultrasound data analysis, one-way analysis of variance (ANOVA) followed by Fisher's post hoc test was used.

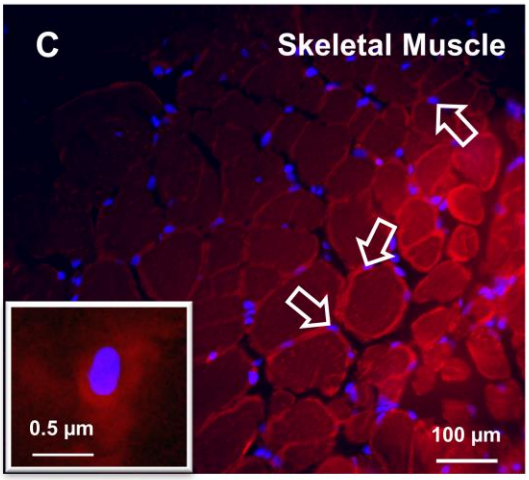
3.3. Results

3.3.1. NUCB1/NLP-Like Immunoreactivity was Detected in Skeletal and Cardiac Muscle in Zebrafish

NUCB1/NLP-like immunoreactivity (DS red; red staining) was detected in the atrial and ventricular cardiomyocytes of zebrafish heart (**Figure 3.1 A and B**). NUCB1/NLP-like immunoreactivity was also detected in the skeletal muscle (**Figure 3.1 C**). DAPI (blue color) stained the nuclei of the cells (**Figure 3.1 A-D**). No staining was detected in negative (preabsorption) control slides (**Figure 3.1 D**).


A
Atrium
0.5 µm
100 µm


B
Ventricle
100 µm


C
Skeletal Muscle
0.5 µm
100 µm

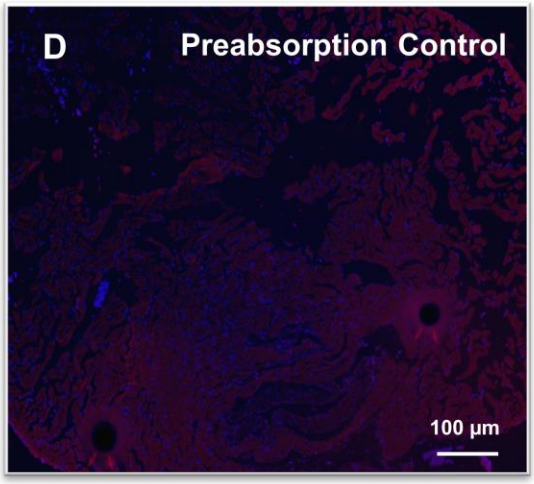

D
Preabsorption Control
100 µm

Figure 3.1. NUCB1/NLP immunoreactivity was detected in atrium and ventricle of heart and skeletal muscle of zebrafish. Nuclei are stained blue (DAPI) and NUCB1 was stained in DS Red. Images were taken at 40X magnification and scale bar = 100 μm (and 0.5 μm for inset). Preabsorption controls were used to confirm the specificity of the antibody (d).

3.3.2. NLP Downregulates Cardiovascular Physiology in Zebrafish and Goldfish

Intraperitoneal administration of NLP (10, 100 ng/g B.W) decreased end diastolic, end systolic, and stroke volumes in zebrafish (F value – 17.36; *P value – 0.041; **P value – 0.0072) (**Figure 3.2 A-C**). NLP (10, 100 ng/g B.W) also decreased heart rate and cardiac output in zebrafish (F value – 25.17; [a]P value – 0.034; P value – **0.009; ***P value – 0.0005) (**Figure 3.2 D, E**). Administration of rat NLP decreased (10 ng/g B.W) end diastolic volume, systolic volume and stroke volume in zebrafish (P value – 0.039) (**Figure 3.3 A-C**). Rat NLP (10 ng/g B.W) decreased heart rate and cardiac output in zebrafish (P value – 0.03) (**Figure 3.3 D, E**). In goldfish, administration of zebrafish NLP (10 ng/g B.W) and rat NLP (10 ng/g B.W) influenced cardiac functions in a manner similar to that seen in zebrafish (P value – 0.043) (**Figure 3.4 A-E**). No significant changes in cardiac functions were observed in response to 10 ng/g B.W NLP scrambled administration in goldfish (P value – 0.17) and zebrafish (P value – 0.098) (**Figure 3.3, 3.4 A-E**).

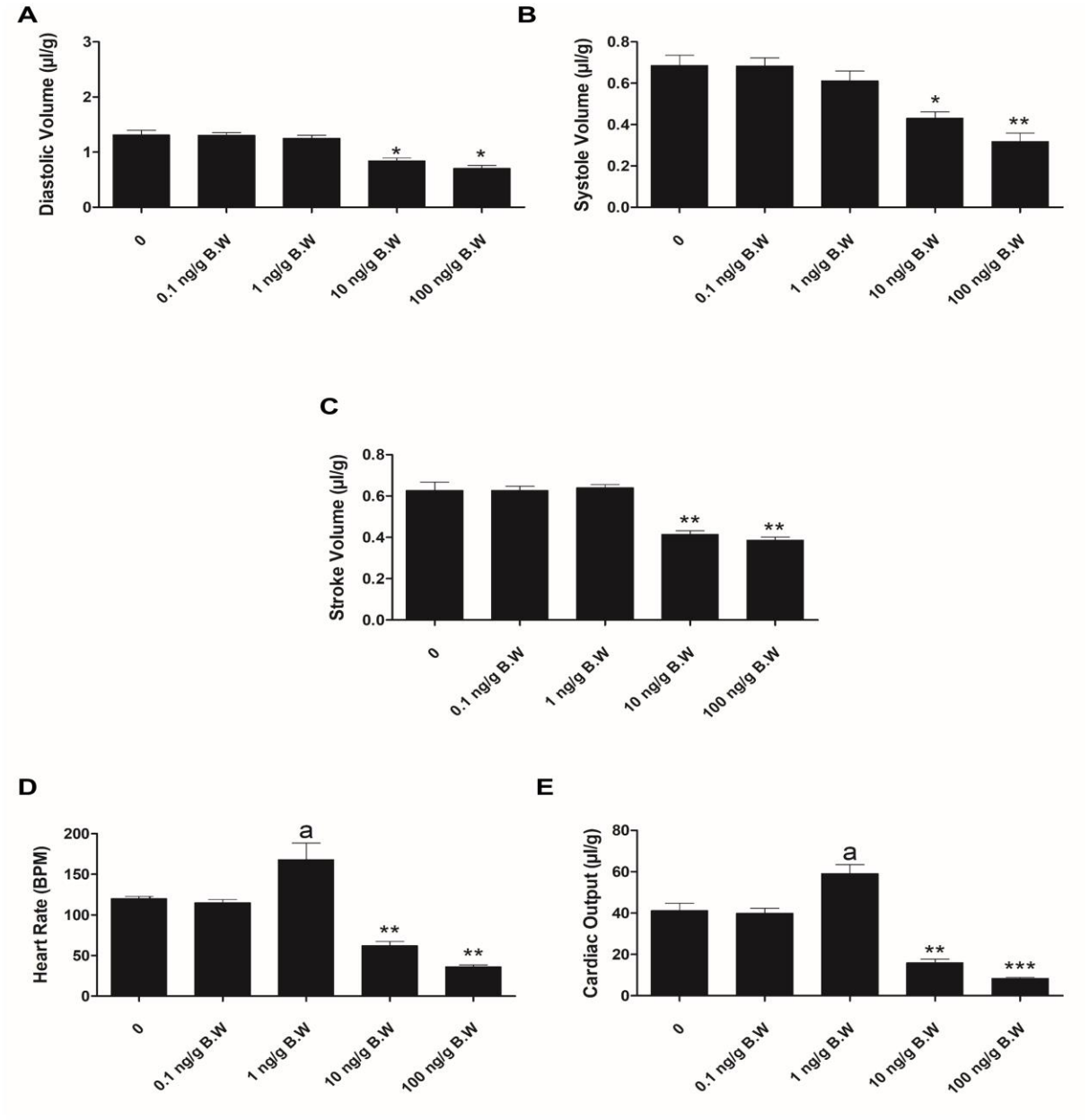

A
Diastolic Volume (µl/g)
3
2
1
0
*
*
0
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
100 ng/g B.W
B
Systole Volume (µl/g)
0.8
0.6
0.4
0.2
0.0
*
**
0
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
100 ng/g B.W
C
Stroke Volume (µl/g)
0.8
0.6
0.4
0.2
0.0
**
**
0
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
100 ng/g B.W
D
Heart Rate (BPM)
200
150
100
50
0
a
**
**
0
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
100 ng/g B.W
E
Cardiac Output (µl/g)
80
60
40
20
0
a
**
***
0
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
100 ng/g B.W

Figure 3.2. NLP administration decreased cardiovascular function in zebrafish. Groups include: Control (0); Dose of Zebrafish NLP (0.1, 1, 10, 100 ng/g B.W). Units are displayed in the Y axis and different cardiac parameters were analyzed. Asterisks denote significant inhibition between control (saline group) and NLP injected groups (**$p<0.01$, n = 6 fish/group) and letters denote significant increase between control (saline group) and NLP injected groups (^{a} $p<0.05$, n=6 fish/ group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

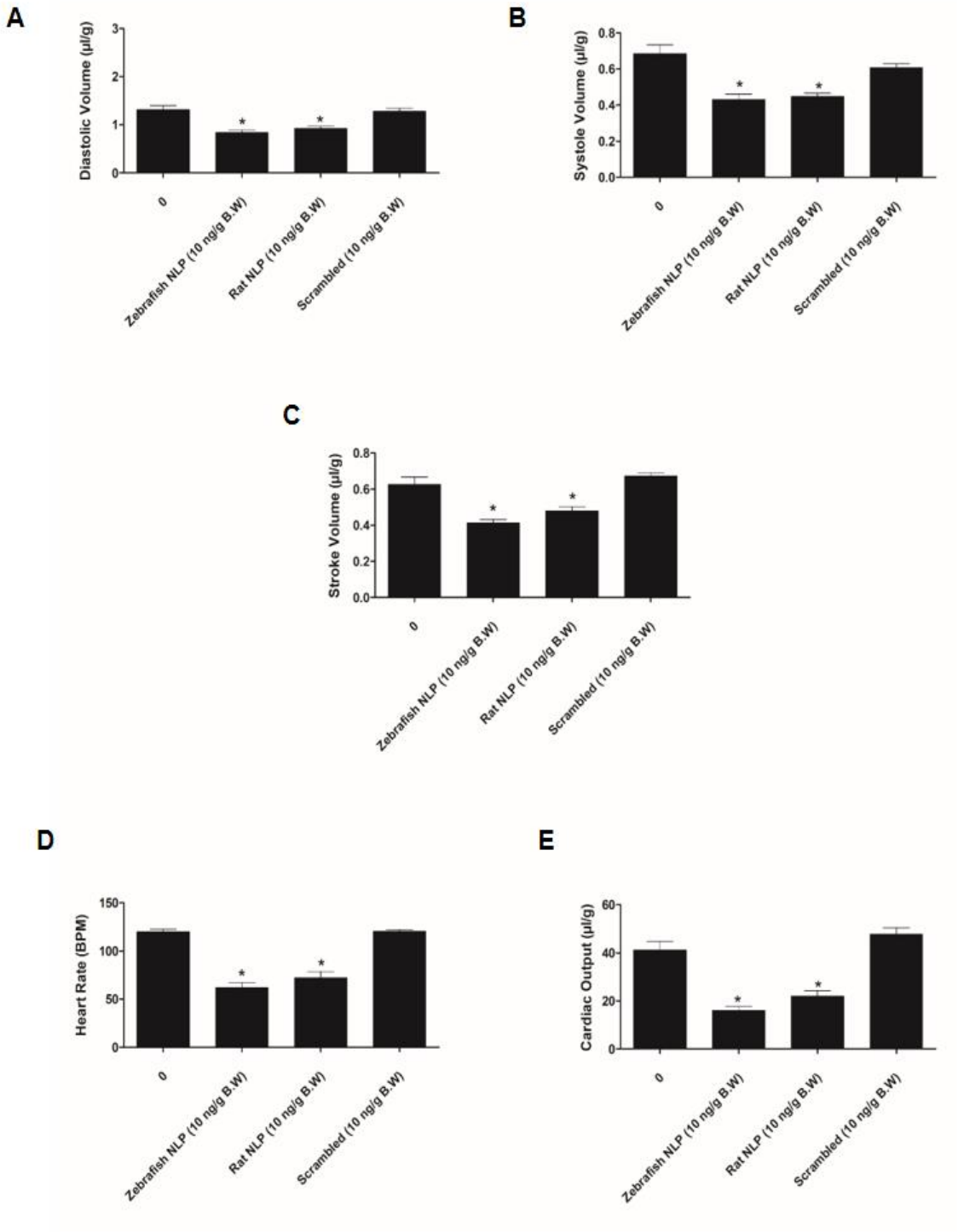

A
Diastolic Volume (µl/g)
3
2
1
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
B
Systole Volume (µl/g)
0.8
0.6
0.4
0.2
0.0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
C
Stroke Volume (µl/g)
0.8
0.6
0.4
0.2
0.0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
D
Heart Rate (BPM)
150
100
50
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
E
Cardiac Output (µl/g)
60
40
20
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 3.3. Zebrafish NLP and rat NLP decreased cardiovascular functions in zebrafish. Groups include: Control (0); Dose of Zebrafish NLP (10 ng/g B.W); Rat NLP (10 ng/g B.W); Scrambled (10 ng/g B.W). Units are displayed in the Y axis and different cardiac parameters were analyzed. Asterisks denote significant differences between control (saline group) and NLP injected groups (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

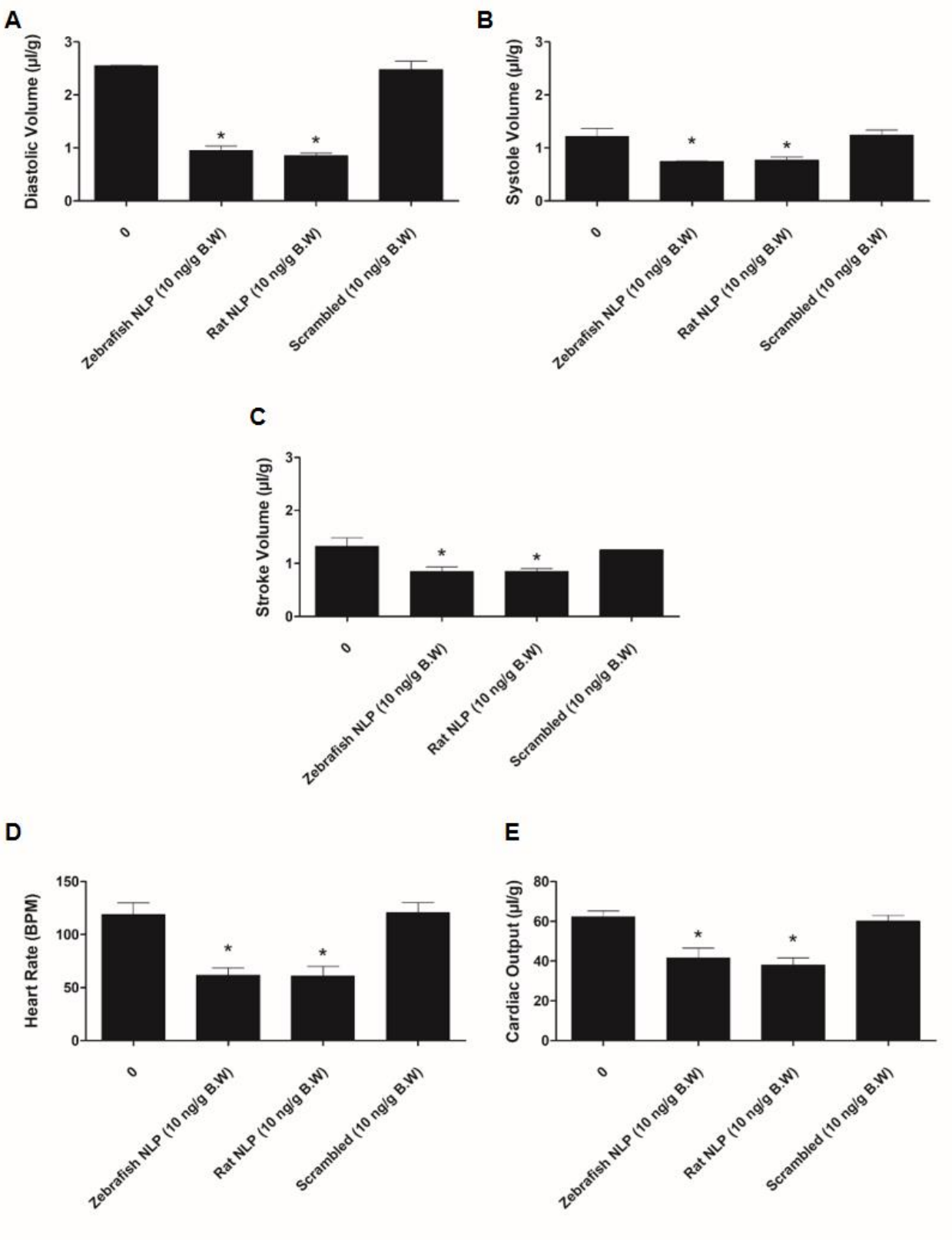

A
Diastolic Volume (µl/g)
3
2
1
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
B
Systole Volume (µl/g)
3
2
1
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
C
Stroke Volume (µl/g)
3
2
1
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
D
Heart Rate (BPM)
150
100
50
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
E
Cardiac Output (µl/g)
80
60
40
20
0
*
*
0
Zebrafish NLP (10 ng/g B.W)
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 3.4. Zebrafish NLP and rat NLP decreased cardiovascular functions in goldfish. Groups include: Control (0); Dose of Zebrafish NLP (10 ng/g B.W); Rat NLP (10 ng/g B.W); Scrambled (10 ng/g B.W). Units are displayed in the Y axis and different cardiac parameters were analyzed. Asterisks denote significant differences between control (saline group) and NLP injected groups (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

3.3.3. Administration of NLP Reduced Irisin mRNA Expression in a Dose-Dependent Manner

Administration of zebrafish NLP (10, 100 ng/g B.W) downregulated irisin mRNA expression in heart (F value – 26.64; P value – 0.012) and muscle (F value – 22.22; P value – 0.018) of zebrafish (**Figure 3.5 A, B**). Administration of rat NLP (10 ng/g B.W) downregulated irisin mRNA relative mRNA expression in the heart (P value – 0.04) and skeletal muscle (P value – 0.035) of zebrafish (**Figure 3.5 B**). Administration of NLP scrambled (10 ng/g B.W) did not affect irisin mRNA expression in zebrafish muscle (P value – 0.2) and heart (P value – 0.28) (**Figure 3.5 B**). Irisin (0.1, 1. 10 ng/g B.W) downregulated NUCB1 mRNA expression (**Figure 3.5 C**) in zebrafish heart (P value – 0.019) and muscle (P value – 0.029).

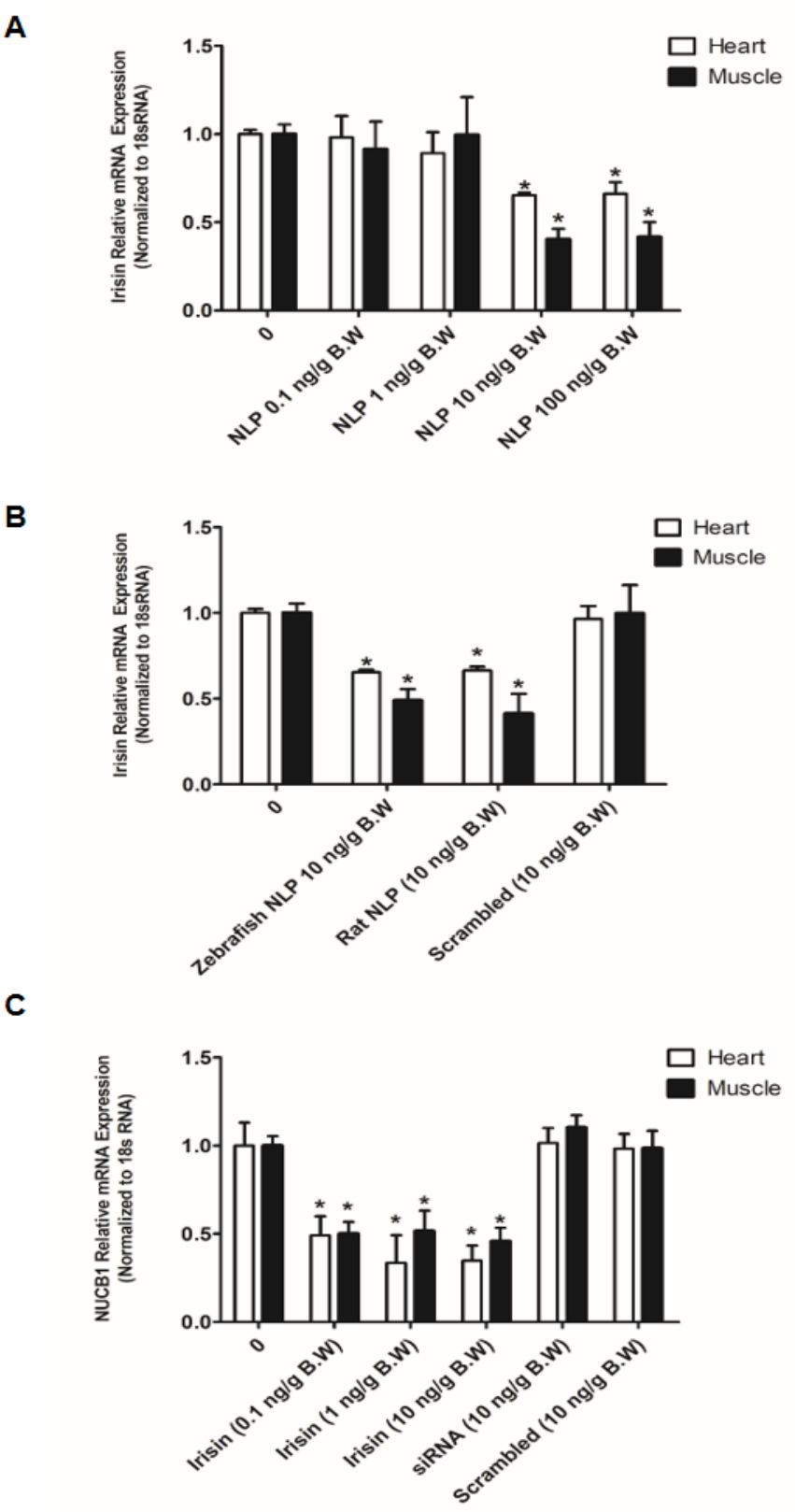

A
Heart
Muscle
Irisin Relative mRNA Expression
(Normalized to 18sRNA)
1.5
1.0
0.5
0.0
0
NLP 0.1 ng/g B.W
NLP 1 ng/g B.W
NLP 10 ng/g B.W
NLP 100 ng/g B.W
B
Heart
Muscle
Irisin Relative mRNA Expression
(Normalized to 18sRNA)
1.5
1.0
0.5
0.0
0
Zebrafish NLP 10 ng/g B.W
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
C
Heart
Muscle
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
1.5
1.0
0.5
0.0
0
Irisin (0.1 ng/g B.W)
Irisin (1 ng/g B.W)
Irisin (10 ng/g B.W)
siRNA (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 3.5. NLP administration downregulated irisin relative mRNA expression in heart and skeletal muscle of zebrafish. Groups include: Control; Dose of Zebrafish NLP (0.1, 1, 10, 100 ng/g B.W); Rat NLP (10 ng/g B.W); Scrambled (10 ng/g B.W) and the relative mRNA expression was normalized to 18s RNA. Asterisks denote significant differences between control (saline group) and irisin and NLP injected groups of the same (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison were used for statistical analysis.

3.3.4. NLP Downregulates RyR1b mRNA and Upregulates ATP2a2a mRNA *In Vivo* in Zebrafish

Zebrafish NLP (10, 100 ng/g B.W) downregulated RyR1b mRNA expression in zebrafish heart (F value – 21.90; P value – 0.016) (**Figure 3.6 A**). Zebrafish NLP (10, 100 ng/g B.W) upregulated ATP2a2a relative mRNA expression in zebrafish heart (F value – 25.8; P value – 0.033) (**Figure 3.6 B**). Administration of rat NLP (10 ng/g) downregulated RyR1b relative mRNA expression (P value – 0.02) and upregulated ATP2a2a mRNA (P value – 0.04) expression in zebrafish heart (**Figure 3.6 C, D**). No effect was found in RyR1b (P value – 1.9) and ATP2a2a (P value – 0.36) mRNA expression in response to NLP scrambled administration when compared to control (**Figure 3.6 C, D**).

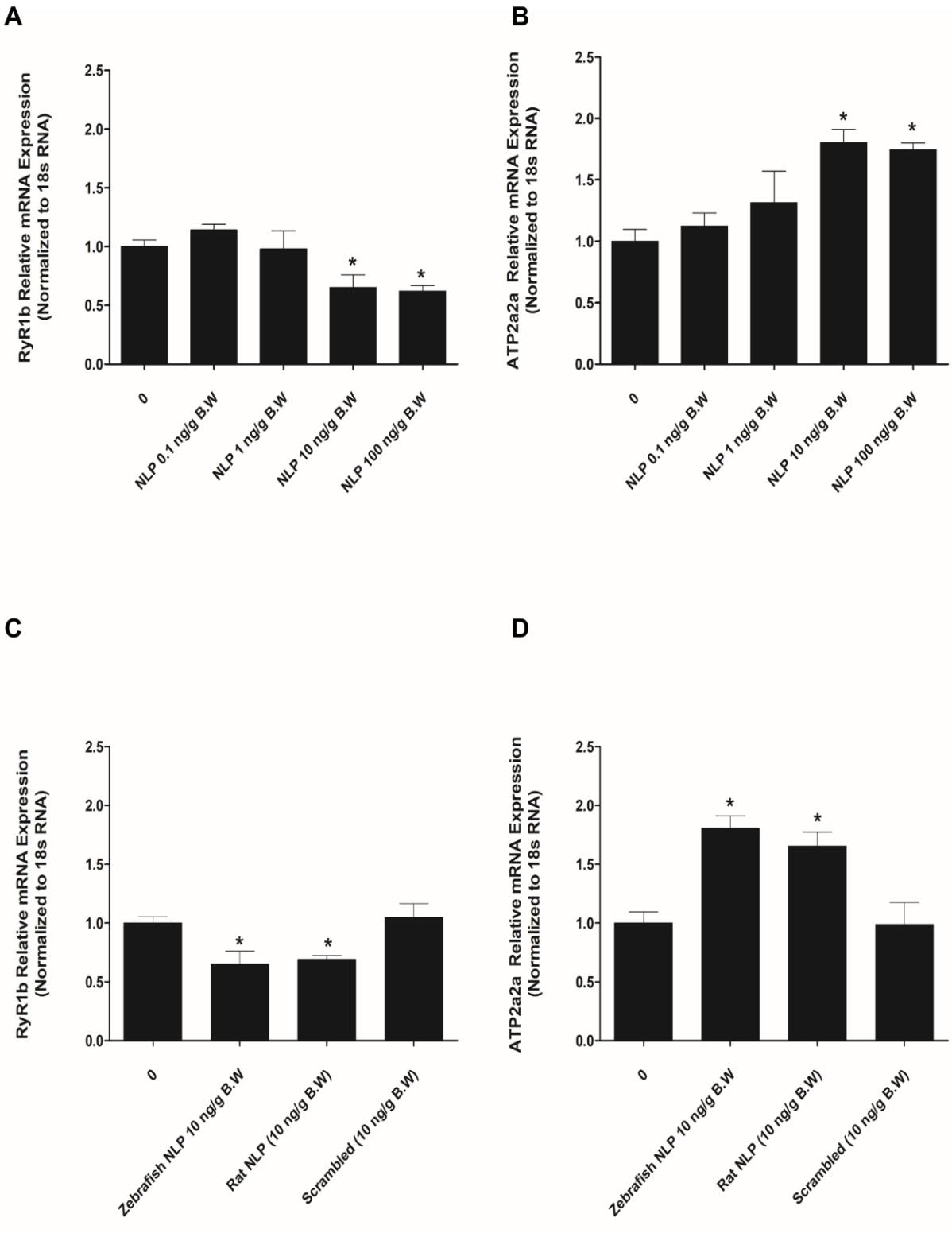

A
RyR1b Relative mRNA Expression
(Normalized to 18s RNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
NLP 0.1 ng/g B.W
NLP 1 ng/g B.W
NLP 10 ng/g B.W
NLP 100 ng/g B.W
B
ATP2a2a Relative mRNA Expression
(Normalized to 18s RNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
NLP 0.1 ng/g B.W
NLP 1 ng/g B.W
NLP 10 ng/g B.W
NLP 100 ng/g B.W
C
RyR1b Relative mRNA Expression
(Normalized to 18s RNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
Zebrafish NLP 10 ng/g B.W
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)
D
ATP2a2a Relative mRNA Expression
(Normalized to 18s RNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
Zebrafish NLP 10 ng/g B.W
Rat NLP (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 3.6. Intraperitoneal injection of zebrafish NLP and rat NLP downregulated RyR1b mRNA and upregulated ATP2a2a mRNA expression in zebrafish heart. Groups include: Control; Dose of Zebrafish NLP (0.1, 1, 10, 100 ng/g B.W); Rat NLP (10 ng/g B.W); Scrambled (10 ng/g B.W) and the relative mRNA expression was normalized to 18s RNA. Asterisks denote significant differences between control (saline group) and NLP injected groups of the same (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison were used for statistical analysis.

3.4. Discussion

We previously published the anorectic action of NLP in fish, and its effects to suppress orexigens and stimulate anorexigens [15]. Here, we report the cardiosuppressive action of NLP and its regulation of calcium handling proteins in fish. Previous results have shown that nesfatin-1 regulated cardiovascular functions and affected calcium handling proteins (ATP2a2a and RyR1b) in zebrafish [21]. NUCB1/NLP-like immunoreactivity was observed in atrial and ventricular cardiomyocytes, and in skeletal muscle of zebrafish (**Figure 3.1 A-C**). The results obtained from our studies are the first line of evidence for NUCB1/NLP expression in cardiac and skeletal muscle of zebrafish. These results suggest that locally produced NUCB1/NLP could contribute to cardiovascular physiology in fish.

I.P administration of NLP elicited cardio suppression in zebrafish and goldfish in a dose dependent manner. Nesfatin-1 is involved in regulating cardiovascular functions in rodents and fish [307-310]. Intravenous injection of nesfatin-1 increased systolic and diastolic blood pressure in rats [311]. ICV injections of nesfatin-1 (60-180 pmol) caused a significant increase in arterial pressure in rats [307]. In zebrafish, intraperitoneal injections of nesfatin-1 (250 ng/g and 500 ng/g B.W) reduced end diastolic volume, cardiac output and heart rate. These results show that nesfatin-1 and NLP elicit similar effects in regulating cardiovascular functions in zebrafish and goldfish. However, the NLP effects on cardiac functions were found at lower dose, compared to the doses tested in studies on nesfatin-1 and cardiovascular biology [21]. It is not feasible to make direct comparisons of dose responsive effects of both fishes without testing lower concentrations of nesfatin-1 and determining its cardiac effects. Considering the high sequence similarity of both NLP and nesfatin-1, it is likely that similar mechanisms mediate the cardiac

effects of both peptides. Differences in the outcomes of rodent and fish studies could be due to differences in route of administration, dosages used, species-specific differences and also mode of action of peptides in organisms tested. Administration of NLP reduced end-diastolic volume post injection in zebrafish and goldfish. The decrease in the end-diastolic volume might be due to increased ventricular wall stiffness or decreased preload controlled by venous pressure [312]. Parasympathetic nervous system could mediate some of the effects of NLP on heart rate and cardiac output upon NLP injection [27, 313]. Another factor that controls cardiovascular functionality is cortisol [314]. Administration of NLP elevated cortisol levels in goldfish (**Appendix A**). Previous results suggest that elevated levels of cortisol resulted in hypertension and increased systolic blood pressure in healthy men [314]. In zebrafish embryos, exposure to high levels of cortisol affected cardiac development and functionality in a dose dependent manner [315]. The potential role of parasympathetic nervous system and stress hormones in mediating NLP effects warrant further studies.

We attempted to understand the possible cellular mechanisms of NLP on cardiac function. Irisin, a skeletal muscle protein has been shown to play an important role in stimulating cardiac function in mice [316]. More recently, irisin has gained importance as a potential biomarker for myocardial infarction due to its abundance in cardiac muscle [39, 237]. ICV injections of irisin increased cardiac output and blood pressure by the activation of hypothalamic neurons in rats [22, 274]. We already reported that irisin has a positive role in regulating cardiac function and modulates muscle proteins in zebrafish [306]. We also determined that irisin regulates cardiovascular function via sympathetic stimulation mediated by β-adrenoceptors in zebrafish (**Chapter 6**). In this study, NLP (10 ng/g and 100 ng/g B.W) downregulated irisin

mRNA expression in zebrafish (**Figure 3.5 A, B**). Meanwhile, administration of irisin (0.1, 1, 10 ng/g B.W) downregulated NUCB1 mRNA expression in zebrafish heart and skeletal muscle (**Figure 3.5 C**). These results indicate that NLP and irisin have opposing effects in regulating cardiac functions in fish. The cardiac effects of NLP appear to be mediated, at least in part, by its negative actions on irisin. Whether other cardioregulatory peptides are also influenced by NLP remains unknown.

Our next studies focused on whether the NLP affects mRNAs encoding key calcium regulatory proteins in heart. Sarco/Endoplasmic Reticulum Ca^{2+} pumps (SERCA pump) play an important role in muscle contraction and relaxation by the uptake and release of calcium ions. Contraction is mediated by the release of calcium with the help of ryanodine receptors, whereas the relaxation occurs by uptake of calcium ions mediated by SERCA pump, stating that SERCA has the ability to restore and release of calcium to maintain contraction-relaxation cycles [202]. Heart failure has been linked to defects in sarcoplasmic calcium uptake and intracellular calcium concentrations, thereby restoring of Ca^{2+} mediated transport system by increasing the expression of SERCA was found to be critical for maintaining cardiac functions [202]. In zebrafish, SERCA protein has been shown to play an important role in cardiac development and function [123, 317]. Expression of SERCA2a/ATP2a2a modulated intracellular calcium levels during ventricular relaxation under different environmental fluctuations in zebrafish [318, 319]. A decrease in ventricular calcium handling by SERCA2a caused a decrease in diastolic and systolic volumes in fish [320]. Another protein of interest is the ryanodine receptor 1b (RyR1b), member of a family of intracellular Ca2+ channels that encodes sarcoplasmic reticulum is known to regulate entry of calcium ions into the cytosol membrane from the intracellular organelles. The

physiological role of RyR is to stimulate the release of Ca2+ during the excitation and coupling. In zebrafish, RyR has been shown to regulate Ca2+ signals during embryonic development in zebrafish. NLP (10, 100 ng/g B.W) injection resulted in an upregulation in ATP2a2a relative mRNA expression in zebrafish heart. The increase in ATP2a2a mRNA expression could be a direct effect of NLP, or a compensatory mechanism in heart tissues as a result of NLP induced cardiosuppressive effects in zebrafish. Contrary to the effects on ATP2a2a, NLP (10, 100 ng/g B.W) injection caused a downregulation of RyR1b mRNA expression in zebrafish heart. Similarly, nesfatin-1 (500 ng/g B.W) upregulated ATP2a2a relative mRNA expression in zebrafish heart, while RyR1b mRNA wasn't altered upon nesfatin-1 treatment in zebrafish heart [21]. These results suggest that two important calcium-handling proteins, SERCA2a/ATP2a2a and RyR1b, are modulated by NLP in zebrafish heart. These changes likely contribute to the cardiac effects seen after NLP injection.

In conclusion, NLP is a novel cardio suppressor in zebrafish and goldfish. This research showed that various cardiovascular functions, including end diastolic and systolic volumes, heart rate and cardiac output were decreased by exogenous NLP similar to nesfatin-1 effects in fish [21]. We also found that administration of NLP downregulated irisin, a cardio-stimulatory myokine. In addition, calcium-handling protein encoding mRNAs were found to be novel targets of NLP in regulating cardiac function. Our results provide several insights for future research in studying the role of NLP in cardiovascular physiology in fish and rodents. Among the future directions, cellular mechanism of actions and additional mediators by which NLP regulates cardiac functions in fish are topics that deserve more research.

TRANSITION

The goal of this research chapter is to determine whether irisin influences feeding, and regulates appetite regulatory peptides in zebrafish. As stated earlier, irisin is abundantly expressed in muscle and heart as well as purkinje cells in the cerebellum and neuroglial cells in rodents by immunohistochemical studies. Intra-hypothalamic injection of irisin decreased food intake and upregulated CART and POMC mRNA expression in Sprague-Dawley rats. While irisin is known to regulate food intake, physical activity and energy homeostasis in mammals, its role in regulating the appetite regulatory proteins are unknown in fish. In goldfish, peripheral injection of irisin inhibited feeding and upregulated CART and orexin-A mRNA expression in fish. Using zebrafish, we determined the possible role of endogenous irisin and exogenous irisin on food intake and the expression of appetite regulatory peptides. This is the first study that has focused on the role of irisin on feeding in zebrafish. Collectively, the results for the first time show a role for endogenous role of irisin in regulating food intake and appetite regulatory peptides in zebrafish.

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Chapter 4

Small Interfering RNA Mediated Knockdown of Irisin Suppresses Food Intake and Modulates Appetite Regulatory Peptides in Zebrafish

4.1. Introduction

Neurotransmitters and neuropeptides regulate feeding and energy homeostasis in vertebrates [17, 54, 55]. Peripheral signals such as adipokines play an integral role in regulating homeostatic signalling pathways in mammals [321]. Hormones primarily act on the hypothalamus, the main metabolic control center of the brain [17]. In the hypothalamus, the expression of several neuropeptides including orexin-A and agouti-related peptide (AgRP) results in the stimulation of food intake, whereas cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) expression decreases food intake [322, 323]. In addition to these appetite regulatory peptides, adipokines play an important role in energy expenditure, partly by the modulation of sympathetic tone, involving the autonomic nervous system [324, 325]. In addition to adipose tissue, the gastrointestinal tract is a major source of metabolic peptides [17], including ghrelin [17, 54, 55]. Irisin is an example of a novel, muscle-derived metabolic regulator [39].

Irisin, a 23 kDa myokine, is primarily expressed in skeletal and cardiac muscles of rodents and humans [39]. Fibronectin type III domain containing 5 (FNDC5) is a 212 amino acid precursor of irisin, in which, amino acids 32-138 correspond to irisin [46]. FNDC5 is

abundant in muscle. During exercise, irisin levels increase, coinciding with an increase in energy expenditure in mammals [39]. Irisin secretion from cardiac and skeletal muscles is increased upon activation of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1 alpha) [46]. Circulating levels of irisin are ~3.6 ng/mL in resting individuals, while it is ~4.3 ng/mL in those undergoing exercise [46]. Processing of FNDC5 by PGC-1 alpha triggers the release of irisin into circulation [39, 46]. Irisin is abundantly expressed in muscle [47, 210]. FNDC5 mRNA is also expressed in other mammalian tissues including rectum, brain, adipose tissue and pericardium [232, 233]. It has been reported that irisin is present in the cerebrospinal fluid and expressed in the hypothalamus, adipose tissue and skeletal muscle in humans [236, 272, 273]. The cell specific expression of irisin was detected in skeletal and cardiac muscles as well as Purkinje cells in the cerebellum and neuroglial cells in rodents by immunohistochemical studies [238, 326]. Elevated levels of circulating irisin induced expression of thermogenin in white adipose cells, leading to browning of white adipocytes and increased thermogenesis [236]. Overexpression of irisin increased energy expenditure, reduced body weight, improved glucose tolerance, stimulated insulin resistance, lipid metabolism and glucose utilization and inhibited insulin resistance in obese C57BL/6 mice [39, 275, 276]. In zebrafish, irisin promotes angiogenesis and modulates matrix metalloproteinase activity through the ERK signaling pathway [47]. Overall, irisin has several biological actions in vertebrates.

While irisin is known to regulate food intake, physical activity and energy homeostasis in mammals [327], its appetite regulatory effects are controversial. In non-obese, non-diabetic, Sprague-Dawley rats, intra-hypothalamic injection of irisin (50-200 nmol/L) decreased food

intake and stimulated CART and POMC mRNA expression [48]. Conversely, infusion of irisin (10 and 100 nM) using osmotic mini pumps into the lateral ventricle increased food intake, plasma ghrelin levels, and NPY mRNA by directly or indirectly affecting uncoupling protein 2 (UCP2) mRNA levels, whereas it decreased plasma leptin levels and POMC mRNA levels in the hypothalamus of non-obese, non-diabetic Wistar Albino rats [328]. In goldfish, intraperitoneal injections of irisin (100 and 250 ng/g) inhibited food intake and increased CART and orexin-A mRNA expression in the brain [49]. Neurotrophic factors such as brain-derived neurotropin factor (BDNF) are very important in regulating feed intake and control of body weight in mice [329, 330]. In mice, BDNF has been shown to influence neuronal development and maintain brain functions by activating neurotransmitters to modulate feed regulation and behavior [330]. Whether BDNF exerts similar biological function in modulating feeding is unknown. Using zebrafish [331] as the model, we determined the possible role of endogenous irisin, and irisin administration on food intake and the expression of appetite regulatory peptides. First, we characterized the tissue specific expression of irisin in zebrafish. Second, we elucidated whether irisin administration or irisin suppression has any effects on food intake of zebrafish. Finally, we studied irisin effects on the expression of mRNAs encoding appetite regulatory peptides in zebrafish. The results of this research, for the first time, show a role for endogenous irisin in regulating food intake and appetite regulatory peptides in zebrafish.

4.2. Materials and Methods

4.2.1. Animals

Zebrafish (*Danio rerio*; 2-4 months old; body weight: ~1 g; males and females) were purchased from Aquatic Imports (Calgary, Canada) and maintained at 27° C under 12L:12D photoperiod cycle. All fish were fed once a day up to 3-4% body weight with slow sinking pellets (slow-sinking pellets; Aqueon, Catalog # 06053) daily at the same time. Fish were euthanized using 0.5% TMS-222 followed by spinal transection. All animal studies complied with the policies of the Canadian Council for Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board.

4.2.2. *In Silico* Analysis

Irisin sequences from various species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The signal peptide site in the zebrafish irisin sequence was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The GenBank Accession numbers of sequences used are provided in figure legends.

4.2.3. Irisin mRNA Expression Detection by RT-PCR and Gel Electrophoresis, and Quantification using RT-qPCR

Fish were anesthetized using 0.5% TMS-222 before dissection and sample collection. Samples of heart and skeletal muscle were collected from zebrafish (n=7), and stored at -80° C. Total RNA was extracted using TRIzol RNA extraction reagent (Catalog # 15596-026,

Invitrogen). cDNAs were synthesized using iScript cDNA reverse transcription supermix (Bio-Rad Laboratories, Catalog # 170-8841). Primers used for RT-qPCR are presented in **Table 4.1**. The primers were validated and optimized for efficiency and annealing temperatures. Real-time quantitative PCR was carried out using iQ SYBR Green supermix (Bio-Rad, Catalog # 170-8880) and CFX Connect Optics module system (BioRad, Canada) controlled by CFX Connect PC-based software (BioRad, Canada) and was analyzed using the Livak method described earlier [21, 280, 294]. Relative mRNA expression of genes of interest were quantified and normalized to the expression of elongation factor 1α (EF1α) (tissue distribution) or 18s RNA (tissue culture studies, food intake studies, siRNA gene knockdown).

4.2.4. Intraperitoneal Injection of Irisin

Irisin sequences of zebrafish and humans have 80% amino acid sequence identity. Due to this, human irisin was injected intraperitoneally (i.p.) into zebrafish at the regular feeding time. Synthetic human irisin (Catalog # 067-16; ≥95% pure, Phoenix Pharmaceuticals Inc., Burlingame, CA) was injected (n = 8 fish; 50 microliters) at 0.1, 1, 10 and 100 ng/g body weight (B.W) just prior to their scheduled feeding time (10 AM). Fish in the control group (n = 8) was injected with 0.9% sodium chloride (saline). Immediately after the administration of irisin or saline, fish were allowed to recover and were fed a pre-weighed quantity of food, and the food recovered 1 h post-administration was dried overnight at 60 °C, to quantify food intake.

4.2.5. Irisin Modulation of Appetite Regulatory Peptides in Zebrafish

Zebrafish (n=8/group) were maintained as described earlier. Post-administration of irisin (0, 0.1, 1 and 10 ng/g B.W), zebrafish were euthanized, brain and gut, as well as heart and skeletal muscle, were collected and processed and RT-qPCR were conducted as described earlier. Tissues collected were used for studying the expression of appetite regulatory peptides preproghrelin, orexin-A and CART mRNAs and were normalized to 18s RNA (housekeeping gene) to determine the effect of appetite regulatory peptides in zebrafish upon irisin administration (**Table 4.1**).

Table 4.1. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Gene Accession Number
	Forward	Reverse		
FNDC5b	GCTTATATCT TCGCGTCCTC	GCCAGTTTCT CTGACTCTTT	59	NM_001044337.1
β-Actin	CTACTGGTA TTGTGATGGACT	TCCAGACAGAG TATTTGCGCT	59	AF057040.1
18s	GGATGCCCT TAACTGGGTGT	CTAGCGGCGC AATACGAATG	60	KY486501.1
Preproghrelin	ATTCAGAGTG TTGTCGTA	AGGAAAGAGC ACATAAGA	56.6	[280]
Orexin-A	GCATATCGG CCGCTTTAATA	GGGTCCTCGAG TCTCTTTCC	60	[280]
CART	GTGCCGAGAT GGACTTTGAC	AGCTGCTTCTC GTTGGTCAG	60	NM_001017570.1
BDNF	GAAGGACGTT GACCTGTATG	ATAGTGCCGC TTGTCTATTC	57	FJ915060.1

4.2.6. Knockdown of Irisin Using siRNA on Food Intake and Appetite Regulatory Peptides in Zebrafish

Zebrafish (n= 6/group) were acclimatized for one week prior to the experiment and were fed with commercial pellet diet (slow-sinking pellets; Aqueon, Catalog # 06053) daily at the same time. The sequences of irisin and scrambled siRNAs are provided in Table 4.2. Zebrafish irisin siRNA was custom synthesized by Dharmacon (Montreal, CA). The control group (saline-injected) (n= 6) were injected with saline (0.9% sodium chloride; Baxter corporation, Catalog # JB1323) (**Table 4.2**). As a negative control, scrambled irisin siRNA was designed to test whether a siRNA of the same length, but with a scrambled sequence based on the irisin mRNA. Scrambled siRNA sequence was designed using GenScript sequence scramble tool (https://www.genscript.com/ssl-bin/app/scramble) and was synthesized by Dharmacon (Montreal, CA) (**Table 4.2**). Zebrafish irisin siRNA and scrambled siRNA were injected i.p. at 10 ng/g B.W. The dose tested was found effective in reducing feeding in a trial, and this was maintained as the desired dose. Immediately after injection, zebrafish were fed, and food intake was quantified as described earlier. Fish were euthanized, brain and gut collected and processed as described earlier. In order to test the effect of siRNA gene silencing strategy, irisin relative mRNA expression was carried out in skeletal muscle and heart tissues respectively and normalized to 18s RNA (housekeeping gene) (**Table 4.1**). Appetite regulatory peptide encoding mRNAs of CART, orexin-A and preproghrelin mRNA in the brain and gut of siRNA treated zebrafish was measured, and data were normalized to 18s RNA expression.

Table 4.2. Forward and reverse sequence of irisin and scrambled siRNA used during gene knockdown studies.

siRNA	siRNA sequence (5'-3')		Length
	Forward	Reverse	
Irisin	C.C.A.A.A.G.A.G.U.C.A.G.A.G.A.A.A.C.U.U.U	P.A.G.U.U.U.C.U.C.U.G.A.C.U.C.U.U.U.G.G.U.U	21
Scrambled	G.C.G.U.A.U.C.A.A.C.G.G.A.G.U.U.A.U.A.U.U	P.U.A.U.A.A.C.U.C.C.G.U.U.G.A.U.A.C.G.C.U.U	21

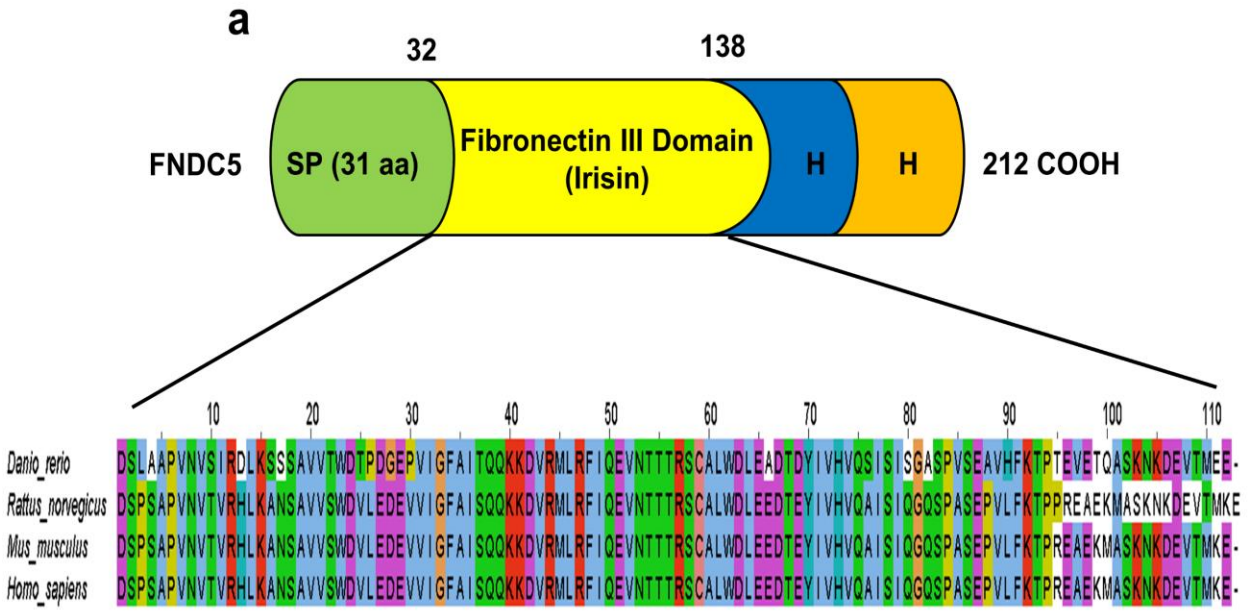
4.2.7. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using PRISM version 5 (GraphPad Inc., USA) and IBM SPSS™ version 21 (IBM, USA) were used for statistical analysis. $P < 0.05$ was considered statistically significant. Data are represented as mean + SEM.

4.3. Results

4.3.1. In Silico Analysis of FNDC5 Sequences and Relative mRNA Expression of Irisin by Gel Electrophoresis

Sequence analysis found a very highly conserved irisin (32-138) in zebrafish (NM_001044337.1). The FNDC5 sequence is 212 amino acids long, of which 32-138 (106 aa) constitutes the irisin sequence. Zebrafish irisin sequence exhibits 70-80% amino acid sequence identity to human, rat and mouse irisin sequences (**Figure 4.1 a**). Abundance of irisin mRNA expression (332 bp) was detected in the skeletal and cardiac muscles of zebrafish (n=6) (**Figure 4.1 b**). No amplicon was detected in other tissues (i.e. brain, liver, gut, ovary and testes) of zebrafish (**Figure 4.1 b**).


a
32
138
FNDC5
SP (31 aa)
Fibronectin III Domain
(Irisin)
H
H
212 COOH
10
20
30
40
50
60
70
80
90
100
110
Danio_rerio
Rattus_norvegicus
Mus_musculus
Homo_sapiens

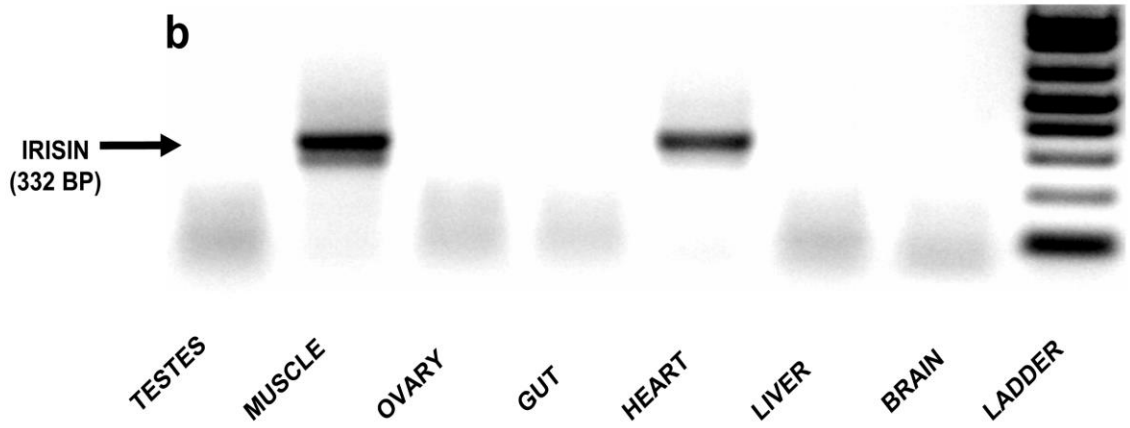

b
IRISIN
(332 BP)
TESTES
MUSCLE
OVARY
GUT
HEART
LIVER
BRAIN
LADDER

Figure 4.1. In silico analysis of FNDC5 sequence and expression of irisin in zebrafish by gel electrophoresis. (a) Schematic representation of FNDC5 sequence showing the irisin peptide (32–138 amino acids) region. The alignment of irisin sequence from various species is shown below. GenBank accession numbers of the sequences used are: Danio rerio (NM_001044337.1), Rattus norvegicus (NM_001270981.1), Mus musculus (NM_027402.4), Homo sapiens (NM_001171941.2). (**b**) Gel electrophoresis of irisin in zebrafish by reverse transcriptase PCR (n = 7).

4.3.2. Intraperitoneal Administration of Irisin Did not Affect Food Intake in Zebrafish

Administration of synthetic irisin by a single intraperitoneal injection did not affect food intake in zebrafish, when compared to control (saline-injected) group (F value – 15.75; P value – 1.87) (**Fig. 4.2**).

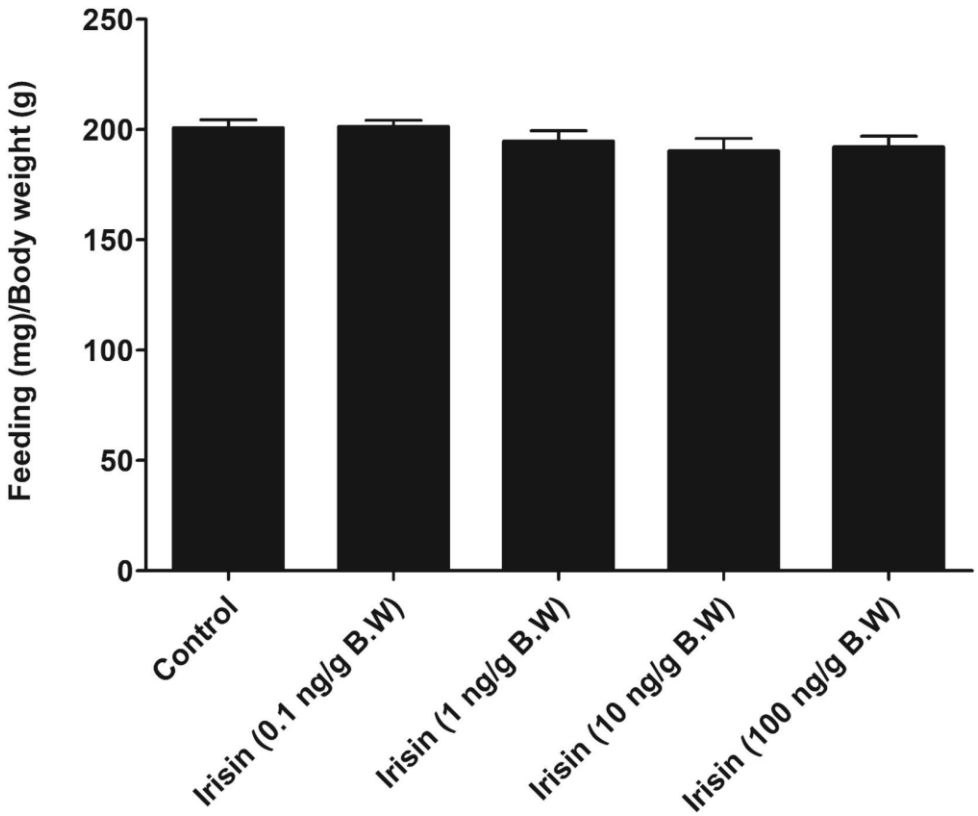


Figure 4.2. Effect of irisin on food intake in zebrafish. Groups include: Control; Dose of Irisin (0.1, 1, 10, 100 ng/g B.W). Asterisks denote significant differences between control (saline-injected) and irisin injected groups in zebrafish (*$p < 0.05$, $n = 6$ fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

4.3.3. Knockdown of Irisin Suppressed Food Intake in zebrafish

Knockdown of irisin by siRNA (10 ng/g B.W) reduced food intake in zebrafish (F value – 34.5; P value – 0,021) (**Figure 4.3 a**). No effect on food intake was observed in response to an intraperitoneal injection of 10 ng/g B.W scrambled irisin siRNA (P value – 1.17) (**Figure 4.3 a**). Intraperitoneal injection of irisin siRNA resulted in the inhibition of irisin mRNA expression by 90% at 10 ng/g B.W in zebrafish heart (F value – 14.67; P value – 0.02) and skeletal muscle (F value – 15.6; P value – 0.035) when compared to control (saline-injected) group (**Figure 4.3 b**). No significant effect on irisin relative mRNA expression was observed in response to an intraperitoneal injection of scrambled irisin siRNA (10 ng/g B.W) in zebrafish heart (P value – 0.75) and skeletal muscle (P value – 0.3), when compared to control (saline-injected) group (**Figure 4.3 B**).

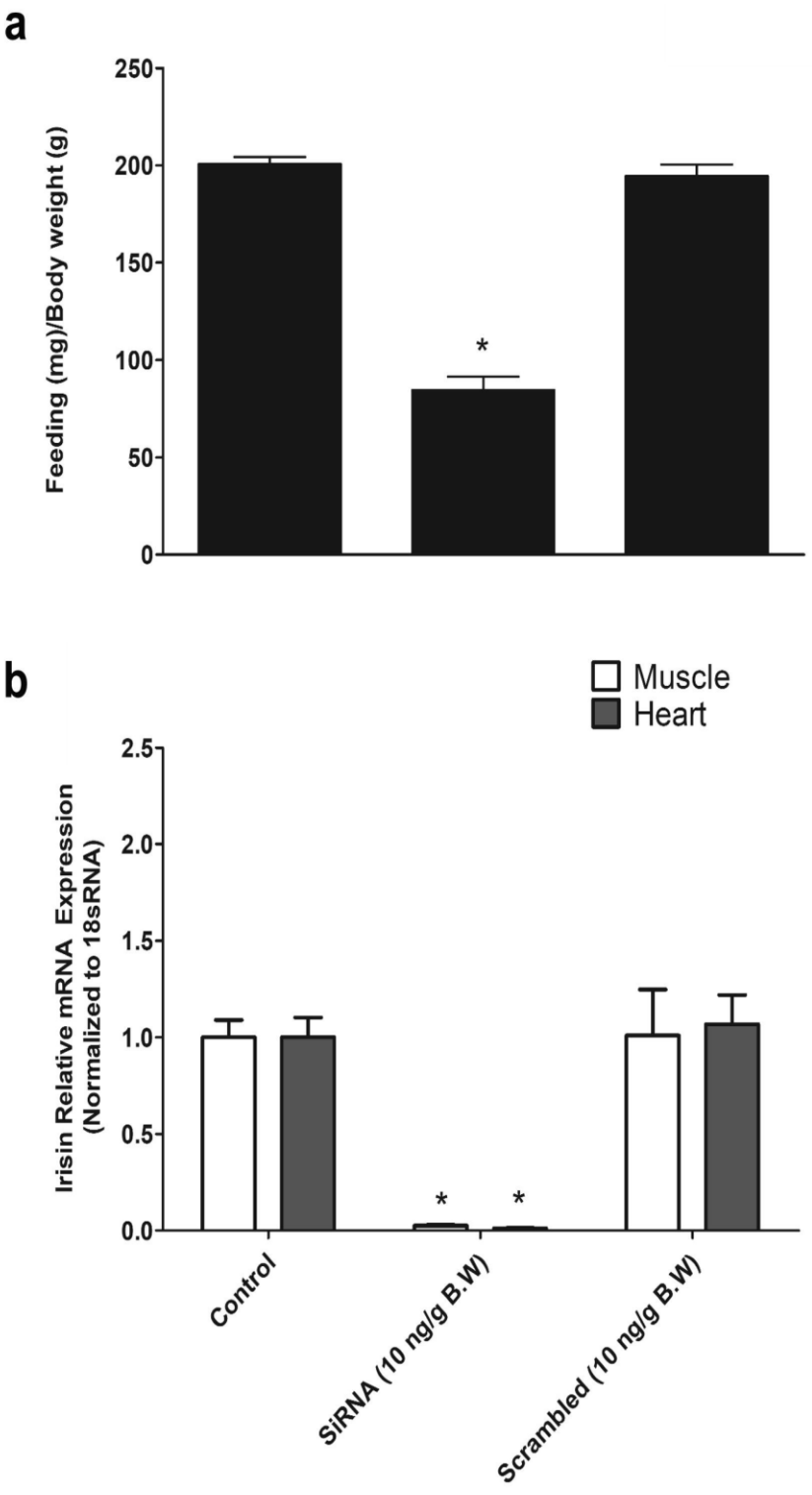

a
Feeding (mg)/Body weight (g)
250
200
150
100
50
0
*
b
Muscle
Heart
Irisin Relative mRNA Expression
(Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
SiRNA (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 4.3. Knockdown of irisin by siRNA suppressed feed intake in zebrafish. Groups include: Control; siRNA (10 ng/g B.W) and scrambled siRNA (10 ng/g B.W). White bars denote muscle and black bars denote heart tissue samples in zebrafish normalized to 18s RNA. Asterisks denote significant differences between control (saline-injected) and irisin injected groups in zebrafish (*$p < 0.05$, $n = 6$ fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

4.3.4. Administration of Irisin Did Not Affect Appetite Regulatory Peptide Encoding mRNAs in Zebrafish

Administration of irisin (0.1, 1, 10 and 100 ng/g B.W) did not affect ghrelin (F value – 2.89; P value – 0.43) **(Figure 4.4 a)** or orexin-A (F value – 3.1; P value – 0.66) **(Figure 4.4 b)** relative mRNA expression in the brain and gut of zebrafish when compared to control (saline-injected) group. Injection of irisin did not affect CART relative mRNA expression in brain (F value – 1.5; P value – 0.7) and gut (P value – 0.12) in zebrafish **(Figure 4.4 c)**.

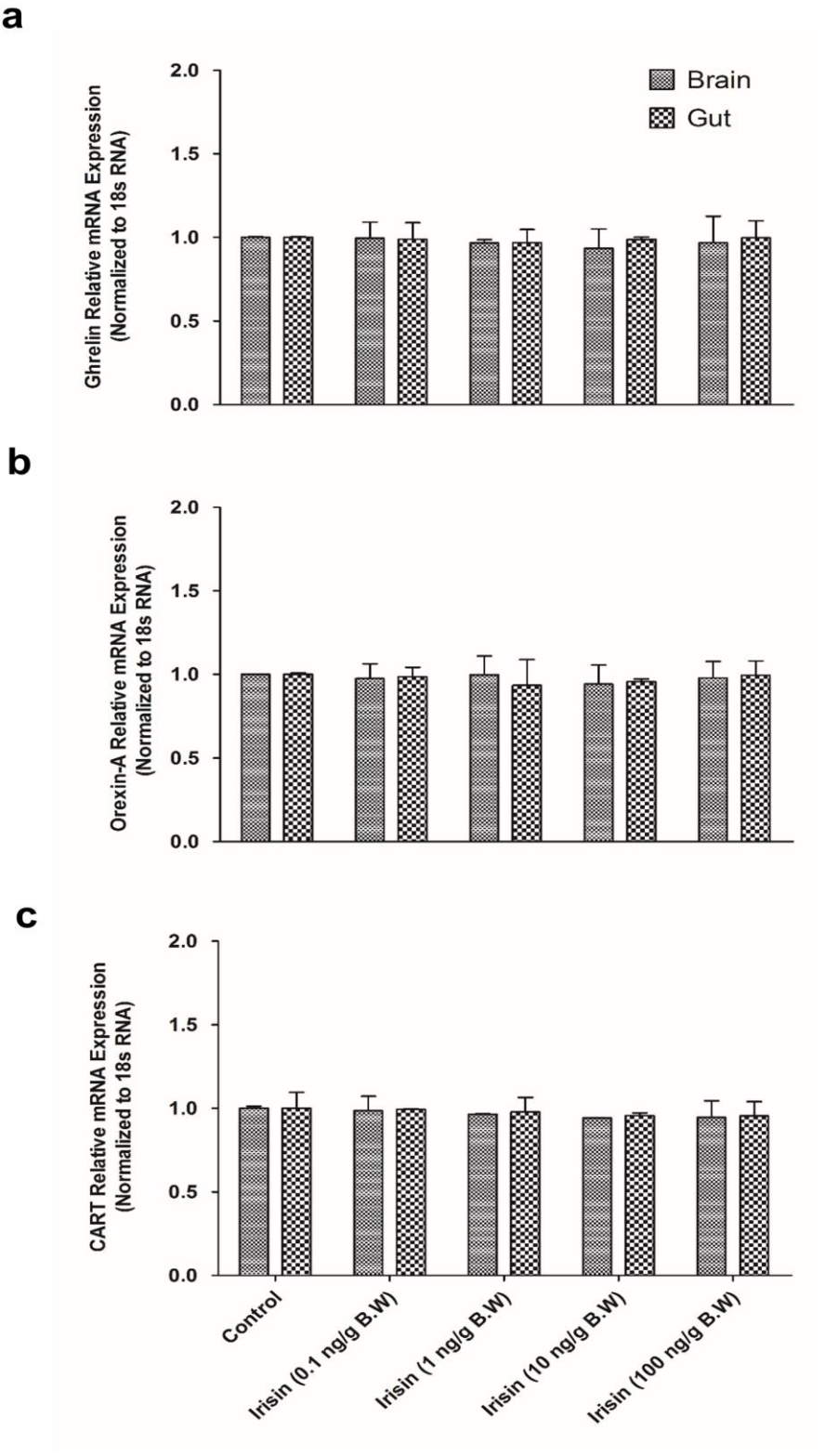

a
Brain
Gut
Ghrelin Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
b
Orexin-A Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
c
CART Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
Control
Irisin (0.1 ng/g B.W)
Irisin (1 ng/g B.W)
Irisin (10 ng/g B.W)
Irisin (100 ng/g B.W)

Figure 4.4. Irisin modulation of appetite regulatory peptides in zebrafish. Groups include: Control; Dose of Irisin (0.1, 1, 10, 100 ng/g B.W) and the relative mRNA expression of ghrelin, orexin-A and CART was normalized to 18s RNA. Data are represented as mean + SEM and normalized to 18s RNA. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

4.3.5. Irisin Knockdown Results in the Downregulation of Orexigens, and Upregulation of CART

Knockdown of irisin (10 ng/g B.W) suppressed preproghrelin (F value – 27.13, P value – 0.036) and orexin-A (F value – 27, P value – 0.025) mRNA expression, and upregulated CART (F value – 27.2; P value – 0.011) mRNA expression in zebrafish brain and gut (**Figure 4.5 a-c**). No effect on mRNAs quantified was observed in response to scrambled irisin siRNA injection (P value – 0.98), when compared to control (saline-injected) group (**Figure 4.5 a-c**). Administration of irisin (0.1, 1, 10 and 100 ng/g B.W) did not affect BDNF relative mRNA expression in brain (P value – 1.23), heart (P value – 0.34), muscles (P value – 0.087) and gut (P value – 0.13) tissues of zebrafish when compared to control (saline-injected) group **(Figure 4.6 a)**. Knockdown of irisin (10 ng/g B.W) resulted in the downregulation of BDNF relative mRNA expression in zebrafish brain (P value – 0.04), heart (P value – 0.018), muscle (P value – 0.033) and gut (P value – 0.04) of zebrafish **(Figure 4.6 b)**. No significant effect on BDNF mRNA expression (P value – 0.5) was observed in response to scrambled irisin siRNA (**Figure 4.6 b**).

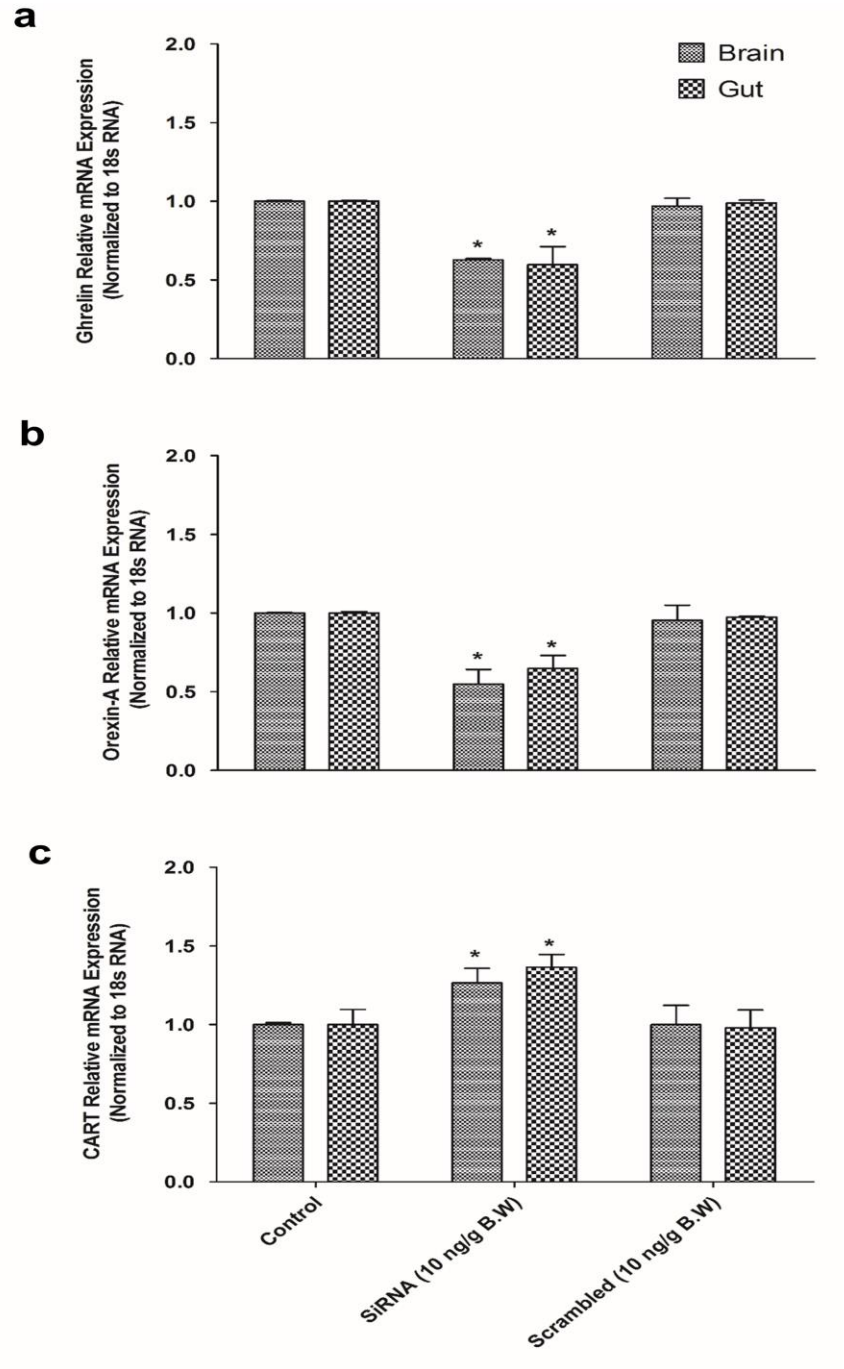

a
Ghrelin Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
Brain
Gut
*
*
b
Orexin-A Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
*
*
c
CART Relative mRNA Expression
(Normalized to 18s RNA)
2.0
1.5
1.0
0.5
0.0
*
*
Control
SiRNA (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 4.5. Knockdown of irisin by siRNA on appetite regulatory peptides in zebrafish. Groups: Control; siRNA (10 ng/g B.W) and Scrambled (10 ng/g B.W). Brain and gut tissues were considered and the relative mRNA expression was normalized to 18s RNA. Asterisks denote significant differences between control (saline-injected) and irisin injected groups in zebrafish (*$p < 0.05$, $n = 6$ fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

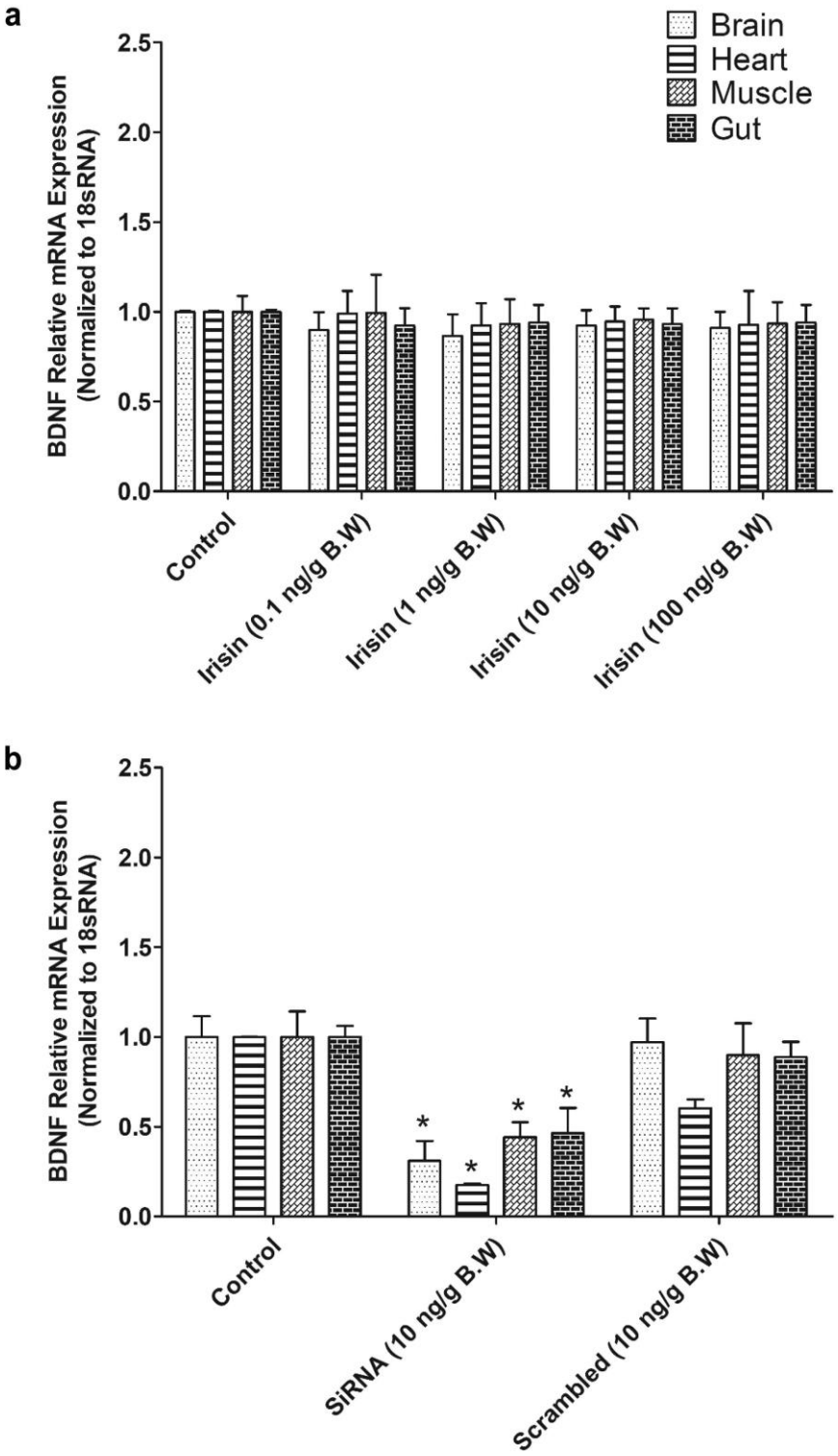

a
Brain
Heart
Muscle
Gut
BDNF Relative mRNA Expression
(Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Irisin (0.1 ng/g B.W)
Irisin (1 ng/g B.W)
Irisin (10 ng/g B.W)
Irisin (100 ng/g B.W)
b
BDNF Relative mRNA Expression
(Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
SiRNA (10 ng/g B.W)
Scrambled (10 ng/g B.W)

Figure 4.6. Modulation of BDNF relative mRNA expression upon knockdown of irisin by siRNA in zebrafish. Groups: Control; Dose of Irisin (0.1, 1, 10, 100 ng/g B.W); siRNA (10 ng/g B.W) and Scrambled (10 ng/g B.W). Brain, heart, muscle and gut tissues were considered and the relative mRNA expression was normalized to 18s RNA. Asterisks denote significant differences between control (saline-injected) and irisin injected groups in zebrafish (*$p < 0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

4.4. Discussion

Irisin is a myokine abundantly expressed in the skeletal muscle and heart of rodents and humans. Our *in silico* analysis of sequences revealed a highly conserved irisin in zebrafish, which shares approximately 80% sequence identity with human, rat and mouse irisin sequences. Here, we report the tissue distribution of irisin in zebrafish, role of endogenous irisin on food intake in zebrafish, and irisin effects on appetite regulatory peptides. Irisin was abundantly expressed in the skeletal muscle and heart of zebrafish. These results agree with a previous study in mice by Bostrom et al [39], which showed abundance of irisin in skeletal and cardiac muscle. We did not see irisin expression in other tissues, while the expression of irisin in brain and liver of mice was reported earlier [231, 238]. These results suggest species specificity in the expression of irisin, suggestive of tissue specific biological actions of irisin. Considering the high sequence identity of irisin sequences and some similarities in its tissue distribution, it is likely that irisin exerts biological actions in zebrafish.

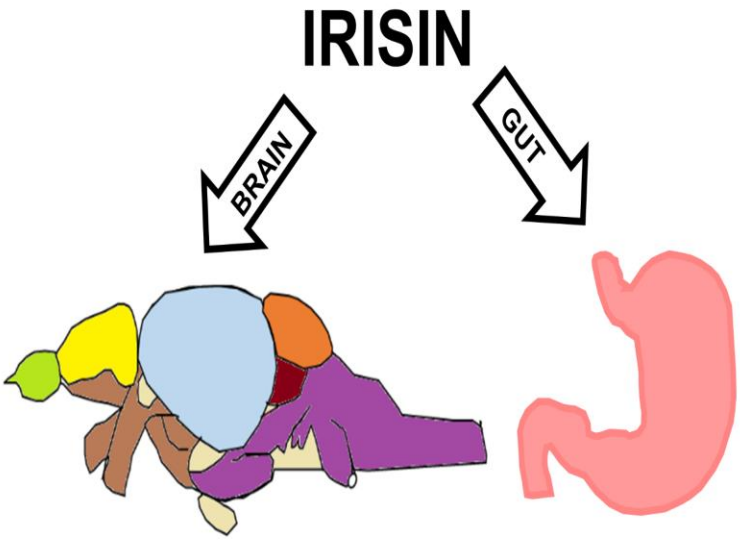
Our next study focused to determine whether irisin has any role in regulating food intake of zebrafish. IP injection of irisin did not affect food intake in zebrafish. In contrast, i.p administration of "mammalian" (species not indicated) irisin at 100 and 250 ng/g body weight doses reduced feeding in goldfish, at 30 min post-injection. Differences in the species used, doses tested, and the duration of food intake monitoring are possible factors that caused this discrepancy. As in the goldfish study [49], which used "mammalian" irisin, and the rat study [328] used central administration of human irisin, we also used non-native peptide in zebrafish studies. The use of a heterologous peptide is a limitation of this study. Future research using the endogenous (fish) form of irisin is essential to determine whether the lack

of effect found in this study is due to the use of a mammalian peptide. On the other hand, knockdown of irisin *in vivo* resulted in inhibition of food intake in zebrafish. Our results presented here are the first line of evidence for a role for endogenous irisin in the regulation of feeding in zebrafish. The decrease in food intake could possibly be an outcome of effects of irisin on other aspects of metabolism, including a decrease in energy expenditure, cardiac functions and physical activity.

siRNA based technologies have been effectively used in zebrafish before, for example, in efficiently silencing the dystropin gene [332]. In another study, microinjection of lamin A, GL2 and eGFP siRNAs into zebrafish embryos resulted in abnormal development and early death of zebrafish embryos [333]. In our study, knockdown of irisin using siRNA had a profound anorexigenic effect in zebrafish. We explored whether irisin siRNA influenced appetite regulatory peptides. Ghrelin [294] and orexin-A [334] are potent orexigens in zebrafish, and CART [298], an anorexigen. Knockdown of irisin resulted in the downregulation of preproghrelin and orexin-A, and upregulated CART mRNA expression in zebrafish brain and gut. These peptides found altered by irisin deficiency are possible mediators of its effects on feeding.

In addition to the appetite regulatory peptides studied, BDNF, a neurotrophin, found to influence neuronal development and maintenance of brain functions by activating neurotransmitters and modulating neurogenesis was also measured [335]. In mammals, BDNF regulates dopamine and serotonin (neurotransmitters) which has been shown to modulate feed regulation and behavior [330]. In humans and goldfish, irisin has been shown to induce

BDNF expression [335]. Our study showed that irisin administration did not affect BDNF mRNA, while siRNA mediated irisin knockdown downregulated BDNF mRNA expression in zebrafish tissues. These results suggest that whole body knockdown of irisin suppresses BDNF, another peptide involved in feeding regulation. Altogether, attenuation of endogenous irisin suppresses various orexigenic peptides, and stimulates anorexigenic peptides to regulate food intake in zebrafish. The mechanisms of action of irisin on food intake, and the pathways that mediate this function require further investigation. **Figure 4.7** summarizes the effects of irisin on feeding and appetite regulatory factors in zebrafish.



	Irisin	Irisin siRNA
Feed Intake	⟷	↓
Orexin-A, Ghrelin	⟷	↓
CART	⟷	↑

↑= Stimulatory effect; ↓ = Inhibitory effect; ⟷ = No effects.

Figure 4.7. Summary of irisin effects on food intake and regulation of appetite regulatory factors in zebrafish. Scheme depicting the role of irisin on feeding and appetite regulatory peptides in zebrafish. Irisin, a myokine is abundantly expressed in cardiac and skeletal muscles in zebrafish. No significant effect on feeding and appetite regulatory peptides was found after the administration of irisin. Knockdown of irisin has a suppressive effect on food intake, downregulated orexin-A mRNA and preproghrelin mRNA, and upregulated cocaine and amphetamine regulated (CART) mRNA expression in zebrafish.

4.5. Conclusion

Research on irisin, especially studies on irisin in non-mammals, is in a state of growth. Our studies elucidated several key aspects of irisin in zebrafish. First, it unraveled a key role for endogenous irisin in regulating food intake in zebrafish. Second, it found that irisin modulates appetite regulatory peptides in fish. While some of our results are in agreement with similar studies in rodents, it appears that species-specific differences exist in irisin actions. Our results establish irisin as a bioactive molecule in zebrafish. It also provides the basis for future research on the mechanisms of action of irisin, and its role in other physiological systems in zebrafish.

TRANSITION

The focus of this chapter was aimed to study the role of irisin on cardiac function and muscle protein regulation in zebrafish. Previous results on irisin reported its endogenous role in regulating feed intake and affecting appetite regulatory proteins in zebrafish. The role of irisin in regulating cardiac physiology in fish is unknown. The main focus of this research was in determining two important aspects of irisin in zebrafish. This is the first study that elucidated irisin's role in cardiac function. The results of this research show a role of irisin in regulating cardiovascular physiology and identified novel targets of irisin in zebrafish.

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Chapter 5

Irisin Regulates Cardiac Physiology in Zebrafish

5.1. Introduction

Skeletal muscle constitutes up to 40% of total body weight, and is considered an exercise dependent endocrine organ that constitutes approximately 75% of body proteins [336, 337]. Skeletal muscle regulates cytokines and myokines that exert autocrine and paracrine effects in humans [338-341]. Some of the skeletal muscle derived cytokines, including interleukin-6 have the ability to regulate glucose and lipid levels [342]. Irisin is a recently confirmed, exercise-induced, 23 kDa myokine abundantly expressed in rodent and human skeletal muscle [41]. Irisin is secreted from FNDC5, a 212 amino acid precursor, after the cleavage of its extracellular portion [39, 40]. FNDC5 is regulated by PGC-1 alpha, which forms an integral part of the muscle post-exercise, and causes an increase in energy expenditure in mammals [39]. Processing of FNDC5 by PGC-1 alpha triggers the release of irisin into circulation [39, 46]. FNDC5 mRNA is expressed in the brain, adipose tissue, gut (rectum) and pericardium in humans [232]. Previous results have reported that irisin is present in the cerebrospinal fluid and is expressed in the hypothalamus, adipose tissue and skeletal muscle in humans [272, 273]. Elevated levels of circulating irisin induced expression of thermogenin in white adipose cells, led to browning, and resulted in increased thermogenesis and energy expenditure [236]. Lower expression of FNDC5 has been associated with reduced aerobic performance in humans, contributing to heart failure [236]. Irisin is considered a key promoter in the central nervous system, and it regulates cardiac contractility [22, 343, 344]. More recently, irisin has

gained importance as a potential biomarker for myocardial infarction due to its abundance in cardiac muscle [39, 345]. Irisin exhibits many biological actions in vertebrates.

Immunohistochemical studies revealed irisin immunopositive cells concentrated in skeletal and cardiac muscles, Purkinje cells in cerebellum and neuroglial cells in rodents [238, 326]. Intracerebroventricular administration of irisin in rats resulted in increased blood pressure and enhanced cardiac contractility [22]. On the other hand, peripheral administration (intraperitoneal injection) of irisin, or irisin injection into the cerebellar area of the nucleus ambiguus decreased blood pressure via vagal stimulation in rats [22, 274]. It has been reported that overexpression of irisin increased energy expenditure, reduced body weight, improved lipid metabolism and glucose tolerance, and suppressed insulin resistance in high fat fed mice[39, 275, 276]. In contrast, knockdown of FNDC5 resulted in a suppressive effect on neural differentiation of mouse embryonic stem cells in mouse [277]. In zebrafish, irisin promotes angiogenesis and modulates matrix metalloproteinase activity through the ERK signaling pathway [47]. Whether irisin exerts any effects on metabolism and cardiac function in non-mammals remains unclear.

Muscle, a major source of irisin, is also a reservoir of other metabolically modulated proteins including PGC-1 alpha, myostatin, troponin and tropomyosin. PGC-1 alpha is an important factor that helps muscle adaptation to endurance exercise [208]. In mice, deletion of PGC-1 alpha resulted in reduced muscle functionality and increased inflammation. Myostatin is member of the transforming growth factor beta family and is a secreted signalling mediator that plays an important role in suppressing the conversion of white adipose tissue to beige/brown adipose tissue in humans [346, 347]. In mice, depletion of myostatin resulted in

increased cell mass, decreased body fat deposition, increased insulin sensitivity, increased fat oxidation and protection from obesity[211-213]. In addition to that knockout of myostatin increased the expression of AMPK, PGC1 alpha and FNDC5 leading to activation of browning of fat in mice [213]. Myostatin has a negative effect on satellite cell growth and postnatal myogenesis in zebrafish [214]. Troponin, a complex protein consisting of troponin C, troponin I and troponin T, is abundantly expressed in cardiac and skeletal muscle and plays an important role in muscle contraction [217]. Cardiac troponin C is a primary determinant of cardiac contractility since it is a calcium binding protein that directly mediates responses to the amount of intracellular calcium released in the heart [218]. On the other hand, cardiac troponin T is a key mediator protein that binds the troponin complex to tropomyosin to mediate controlled interaction between actin and myosin filaments in the myocardial cells of the heart [219]. Overexpression of troponin T has resulted in myocardial damage and its release into circulation from damaged cardiomyocytes is currently used as a biomarker for diagnosing acute myocardial infarction [19, 348]. It is possible that myostatins and troponins contribute to irisin effects on cardiac and metabolic physiology.

We hypothesized that irisin has cardiac function and modulates muscular proteins in zebrafish. The main focus of this research was in determining two important aspects of irisin in zebrafish. First, we elucidated whether irisin has any whole animal effects, by examining cardiac function in zebrafish. Second, we studied irisin effects on the expression of muscle proteins discussed above in zebrafish. The results of this research show a role for irisin in regulating cardiovascular physiology and identify novel targets of irisin in zebrafish.

5.2. Materials and Methods

5.2.1. Animals

Zebrafish (*Danio rerio*; 2-4 months old; body weight: ~1 g) were purchased from Aquatic Imports (Calgary, Canada) and were maintained at 27° C under 12L:12D photoperiod cycle. All fish were fed once a day up to 3-4% body weight with slow sinking pellets (slow-sinking pellets; Aqueon, Catalog # 06053). Fish were euthanized using 0.5% TMS-222 followed by spinal transection. All animal studies complied within the policies of the Canadian Council for Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board (2012-0033).

5.2.2. Western Blot Analyses

Total protein samples from heart and skeletal muscle was collected to confirm the presence of irisin by Western blot analysis. Fish (n= 6) were euthanized using 0.5% TMS-222 before dissection and tissue collection. Tissues for Western blot were homogenized using T-PER tissue protein extraction buffer (Thermo Scientific, Catalog # 78510) followed by protein concentration determination by Bradford assay using NanoDrop 2000c (Thermo, Vantaa, Finland). The samples were prepared using 1X Laemmli buffer containing 0.2% of 2-mercaptoethanol (Bio-Rad, Catalog # 161-0737 and -0710) and were subjected to boiling at 95° C for 5 min followed by vortexing prior to loading. Tissue total protein samples (40 µL; 15, 30 or 40 µg total protein from heart and skeletal muscle of normal zebrafish, or siRNA treated zebrafish), or synthetic irisin (positive control; 10 µg) were loaded and were run on a gradient gel (Bio-Rad, Catalog # 456-1104) at 200V for of 30 min. After the run, proteins

were transferred to a 0.2 µm BioTrace nitrocellulose membrane (PALL Life Sciences, Catalog # 27377-000) subjected to blocking using 1X RapidBlock solution (AMRESCO, Catalog # M325). In order to detect the presence of irisin and β-tubulin (reference protein; Catalog # 2146, Cell signalling, Danvers, MA), rabbit polyclonal FNDC5 antibody (Catalog # ab131390, 1:3000, Abcam, Ramona, Massachusetts) for irisin was used. As the secondary antibody, goat anti-rabbit IgG (H+L) HRP conjugate (Catalog # 170-6515, 1:3000, Bio-Rad) was used. For visualization of protein, the membrane was incubated for 5 min in Clarity Western ECL substrate (Bio-Rad, Catalog # 170-5061) and imaged using ChemiDoc MP imaging system (Bio-Rad, Catalog # 170-8280). Membrane stripping for detection of reference protein was conducted using Western blot stripping buffer (Thermo Scientific, Catalog # 46430). Primary antibody was pre-absorbed in 10 µg synthetic human irisin (Catalog # 067-16, Phoenix Pharmaceuticals, Inc, Burlingame, CA) overnight and was used as pre-absorption controls for zebrafish tissues to confirm the specificity of the irisin antibody. Precision plus protein dual Xtra standards (Bio-Rad, Catalog # 161-0377) were used as the marker to detect the molecular weight of irisin and beta-tubulin.

5.2.3. Immunohistochemistry

The localization of the irisin protein in zebrafish heart and skeletal muscle sections were detected by immunohistochemical (IHC) studies as described in detail earlier [21]. The primary antibody used was rabbit polyclonal FNDC5 antibody (Catalog # ab131390, 1:3000, Abcam, Ramona, Massachusetts) for irisin. The slides were then washed with PBS and then were incubated with secondary antibody for one hour at room temperature. Goat polyclonal anti-rabbit IgG (Catalog # TI-1000, 1:500 dilution, Vector Laboratories, California) was used

as secondary antibody for irisin respectively. The slides were then rewashed with PBS and were mounted on Vectashield medium containing DAPI dye (Blue, Vector Laboratories). The slides were dried, and imaged using a Nikon inverted microscope (L100) (Nikon DS-Qi1 MC camera, ON, Canada) and analyzed using NiS Elements imaging software (Nikon, Canada). For controls, primary antibody was pre-absorbed in 10 µg synthetic human irisin (Catalog # 067-16, Phoenix Pharmaceuticals, Inc, Burlingame, CA) overnight to confirm the specificity of the irisin antibody in zebrafish tissues. Tissue sections incubated with secondary antibody alone, or preabsorption using synthetic irisin peptide, were used as negative controls.

5.2.4. Tissue Culture Studies

Samples of heart and skeletal muscle from zebrafish (n=8/group) were collected upon euthanasia using 0.5% TMS-222. Four tissues were added to each plate and were suspended in the fresh media for a period of 2 hours. The media for the tissue culture were prepared containing the following components; DMEM 1X (500 ml); NAHCO3 (1.85g); penicillin/streptomycin (5.5 ml) and gentamicin (250 mg) respectively. Plates were prepared and fresh medium (1 mL) was added to plates and incubated. Different concentrations of irisin (0, 0.1 and 10 nM) were prepared containing the fresh media were prepared accordingly during this incubation period. Post incubation, plates were replaced with media containing irisin at different concentrations. The plates were incubated for 60 or 120 mins respectively. The tissues were then collected and stored at -80° C until further analysis. In order to determine the effect of irisin on muscle proteins, tissues collected were used for studying the relative mRNA expression of troponin C, PGC-1 alpha, myostatin-a and myostatin-b and normalized to 18s RNA (housekeeping gene) (**Table 5.1**). PCR was carried out using iQ

SYBR Green supermix (Bio-Rad, Catalog #170-8880) and CFX Connect Optics module system (Bio-Rad, Canada) controlled by CFX Connect PC-based software (Bio-Rad, Canada).

Table 5.1. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Gene Accession Number
	Forward	Reverse		
FNDC5b	GCTTATAT CTTCGCGTCCTC	GCCAGTTTC TCTGACTCTTT	59	NM_001044337.1
PGC- 1 alpha	TCTATTCG GAAGGGCCCAGA	GGTGGTGCTG TCTCGTTTTG	58	XM_017357139.2
Myostatin-b	TCCTTTAGC ACGCCTTGGAA	TGCTTGAGTC GGAGTTTGCT	60	NM_131019.5
Myostatin-a	TTTTGAGCA TCCTGCGCCTA	ATCTTTGGGCT CAGTGCGAA	60	NM_001004122.2
Troponin-C	GCAGAAAAA TGAGTTCCGTGC	TTCCGCCAGT TCTTCCTCTG	60	AF180890.1
Troponin T2D	AGTTCAGGAGG AAGTGGATGAGT	AGTCTGGCTT GACGCTCTTTC	60	NM_001025179.1
β-Actin	CTACTGGTAT TGTGATGGACT	TCCAGACAGAG TATTTGCGCT	59	AF057040.1
18s	GGATGCCCTTA ACTGGGTGT	CTAGCGGCGC AATACGAATG	60	KY486501.1

5.2.5. Dose Dependent Effects of Irisin on Cardiac Function in Zebrafish

Cardiac function was assessed in zebrafish using a VEVO 3100 high frequency ultrasound machine (Visualsonics, Markham, ON), using B-mode imaging as described earlier [21, 122]. Zebrafish (4 months old) were injected intraperitoneally (6 µL) with synthetic human irisin (Catalog # 067-16; Phoenix pharmaceuticals, Burlingame, CA) at doses 0.1, 1, 10 ng/g body weight. The control group were injected with 6 µL of saline (0.9% sodium chloride). The siRNA sequences of irisin and a scrambled control siRNA are represented in **Table 5.2**. Zebrafish irisin siRNA was synthesized by Dharmacon (Montreal, CA). The control group (n= 6) were injected with saline (0.9% sodium chloride; Baxter corporation, Catalog # JB1323) (**Table 5.2**). Scrambled irisin siRNA was designed to test whether any siRNA sequence that shares same set of mRNAs and length but was highly dissimilar in the arrangement could elicit same biological effect on zebrafish. Scrambled siRNA sequence was designed using GenScript sequence scramble tool (https://www.genscript.com/ssl-bin/app/scramble) from the corresponding zebrafish siRNA sequence and synthesized by Dharmacon (Montreal, CA) (**Table 5.2**). Zebrafish irisin siRNA and scrambled siRNA were injected intraperitoneally at 10 ng/g B.W. Each fish was allowed to recover for a period of 15 min. Zebrafish were anesthetized prior to ultrasound experiments using 20 mg/L Aquacalm (Syndel Laboratories, Canada). Fish were then transferred to a groove in a Styrofoam-lined holding dish and placed ventral side up, with aerated, 27±0.5°C water containing 20 mg/L Aquacalm superfusing the fish to maintain anesthesia throughout ultrasound testing and minimal impact towards cardiovascular functions in zebrafish [21, 122].

A MX700 scan head was used to obtain short and long-axis views of the zebrafish ventricle in B-mode. The areas of three different short axis views along the ventricle were measured as A1, A2, A3 while the ventricular length of long axis view was measured and divided by three to give ventricular height (h) as per equation (1). All of these values were measured at both systole and diastole volume using Visualsonics software (Markham, ON). Using these values, end systolic and diastolic volumes (mm^3 = μl) were calculated for each zebrafish ventricle using the equation:

$$V=(A1+A2)\ h+((A3h)/2)+(\pi/6(h^3)) \quad (1)$$

Stroke volume (SV) was obtained by subtracting end systolic volume from end diastolic volume. Heart rate measurements were calculated by counting the number of heart beats per 10 s during the B-mode ultrasound video loop and converted to beats per minutes (bpm). Cardiac output was measured by multiplying heart rate and stroke volume (SV):

$$\text{Cardiac output} = \text{bpm} * \text{SV} \quad (2)$$

The body weight of the fish was measured, and all cardiac volume and output data were normalized to body weight and analyzed further using statistical analysis.

Table 5.2. Forward and reverse sequence of irisin and scrambled siRNA used during gene knockdown studies.

siRNA	siRNA sequence (5'-3')		Length
	Forward	Reverse	
Irisin	C.C.A.A.A.G.A.G.U.C.A.G.A.G.A.A.A.C.U.U.U	P.A.G.U.U.U.C.U.C.U.G.A.C.U.C.U.U.U.G.G.U.U	21
Scrambled	G.C.G.U.A.U.C.A.A.C.G.G.A.G.U.U.A.U.A.U.U	P.U.A.U.A.A.C.U.C.C.G.U.U.G.A.U.A.C.G.C.U.U	21

5.2.6. Regulation of Muscular Proteins Post Irisin Injection

Zebrafish (n= 8/group; 4 months old) were maintained as described earlier. On the day of the study, synthetic human irisin peptide was intraperitoneally injected (6 µL) at different doses [0, 0.1 ,1 and 10 ng/g body weight (B.W)]. Zebrafish euthanasia, tissue collection and processing were conducted as described earlier. Tissues collected were used for studying the expression of muscular proteins such as troponin C, PGC- 1 alpha, beta-actin, myostatin-a and myostatin-b. Data were normalized to 18s RNA (housekeeping gene) (**Table 5.1**).

5.2.7. Effect of Irisin siRNA on Muscular Proteins in Zebrafish

Zebrafish (n= 6/group; 4 months) were maintained as described earlier. On the day of the experiment, exogenous administration of irisin siRNA and scrambled irisin siRNA were intraperitoneally injected at 0 and 10 ng/g B.W (**Table 5.2**). One-hour post-injection, zebrafish were euthanized using 0.5% TMS-222, spinal transected, and heart and muscle were collected, and stored at –80° C for total RNA extraction. In order to determine the effect of exogenous irisin siRNA on muscular proteins, RT-qPCR were carried out using troponin C, PGC-1 alpha, beta-actin, myostatin-a and myostatin-b. Data were normalized to 18s RNA (housekeeping gene) (**Table 5.1**).

5.2.8. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using PRISM version 5 (GraphPad Inc., USA) and IBM SPSS™ version 21 (IBM, USA) were used for statistical analysis. $P < 0.05$ was considered statistically significant. Data are

represented as mean + SEM. For ultrasound data analysis, one-way analysis of variance (ANOVA) followed by Fisher's post hoc test and data were normalized to the body weight of fish.

5.3. Results

5.3.1. Irisin was Detected in Heart and Muscle of Zebrafish

Western blot analysis detected irisin protein at 23 kDa in zebrafish (2 months old) heart and skeletal muscle (**Figure 5.1 a**). No bands were detected in tissues from zebrafish treated with irisin siRNA (**Figure 5.1 a**). Preabsorbed samples using synthetic irisin did not detect any band in zebrafish tissues (**Figure 5.1 b**). β-tubulin was used as the reference protein (**Figure 5.1 c)**. Beta tubulin was visible in all samples (**Figure 5.1 c)**.

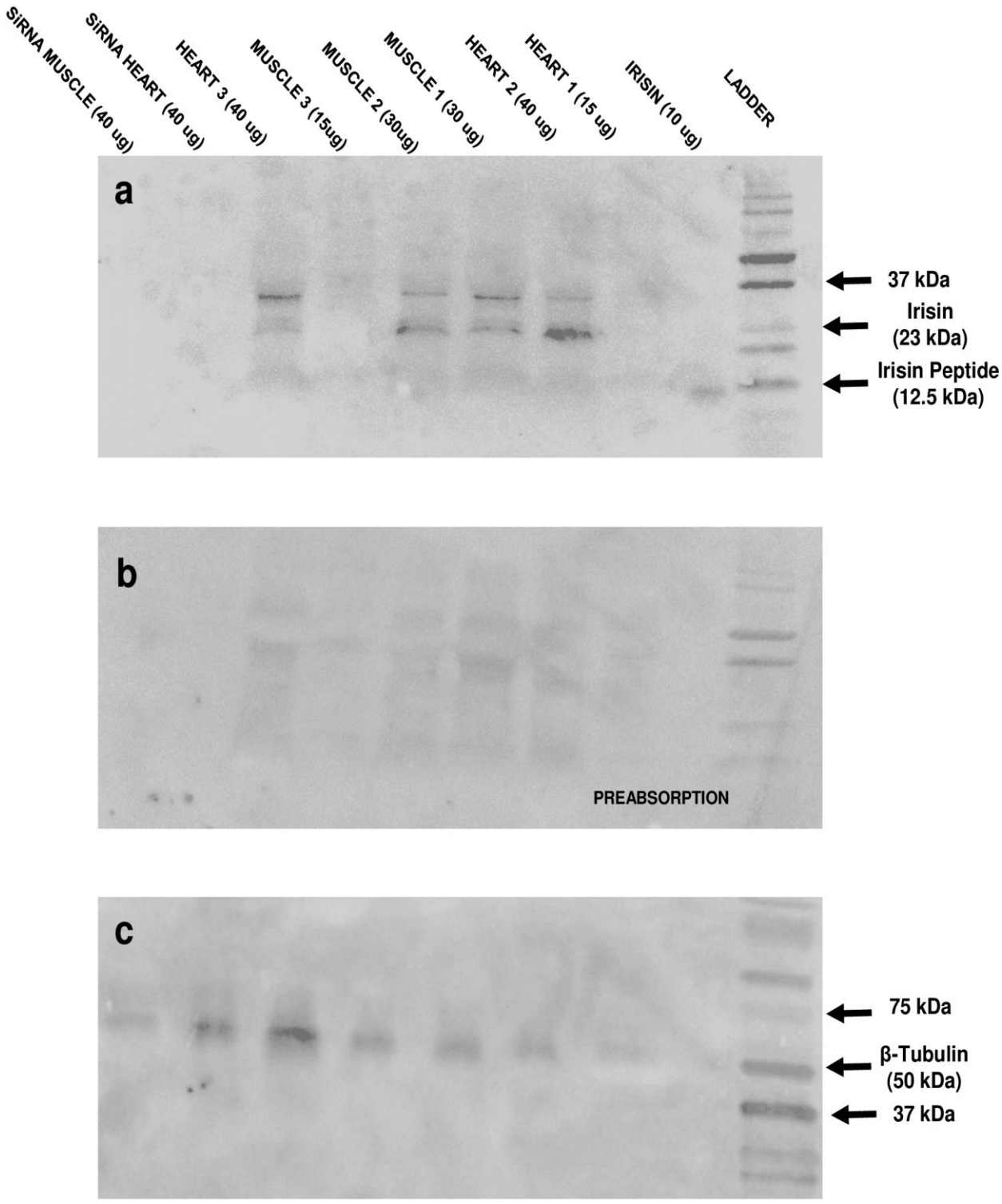

SiRNA MUSCLE (40 ug)
SiRNA HEART (40 ug)
HEART 3 (40 ug)
MUSCLE 3 (15ug)
MUSCLE 2 (30ug)
MUSCLE 1 (30 ug)
HEART 2 (40 ug)
HEART 1 (15 ug)
IRISIN (10 ug)
LADDER
a
37 kDa
Irisin
(23 kDa)
Irisin Peptide
(12.5 kDa)
b
PREABSORPTION
c
75 kDa
β-Tubulin
(50 kDa)
37 kDa

Figure 5.1. Irisin was detected in the heart and skeletal muscle of zebrafish by Western blot analyses. (**a**) Western blot showing irisin in different concentrations of total protein extract from heart and skeletal muscle of untreated zebrafish (tissue name, and concentration of protein loaded in brackets), and siRNA treated zebrafish (labeled siRNA-heart; siRNA-muscle), and synthetic irisin (positive control); (**b**) pre-absorption control using total protein as indicated in A (age: 2 months; n=6 zebrafish). Figure (**c**) shows the internal control (β-tubulin) expression in the above tissues. Western blot detected irisin protein at 23 kDa (**a**) while no protein was detected in the preabsorption control (**b**) in skeletal muscle and heart tissues of zebrafish. A band representing beta tubulin was detected in all tissue samples tested, except for the siRNA treated ones.

5.3.2. Irisin Immunoreactivity was Detected in Skeletal and Cardiac Muscle of Zebrafish

Irisin immunoreactivity (red) was detected in the atrial and the ventricular cardiomyocytes of zebrafish (**Figure 5.2 a-b**). Irisin immunoreactivity was also detected in zebrafish skeletal muscle (**Figure 5.2 c**). DAPI (blue) stained the nuclei of cells (**Figure 5.2 a-d**). No immunoreactivity was observed in sections stained with secondary antibody alone, and in preabsorption controls (**Figure 5.2 d**).

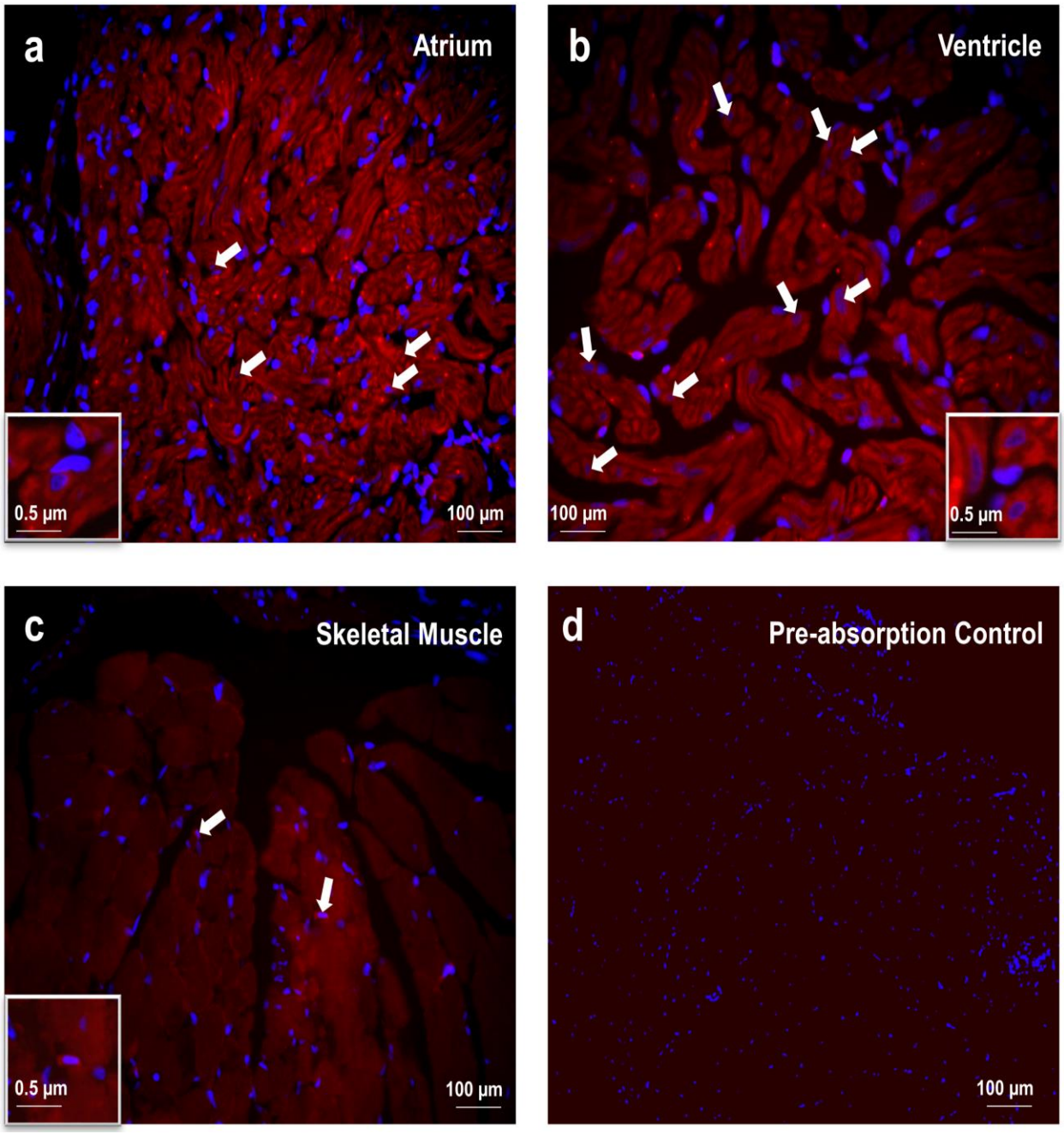

a
Atrium
0.5 µm
100 µm
b
Ventricle
100 µm
0.5 µm
c
Skeletal Muscle
0.5 µm
100 µm
d
Pre-absorption Control
100 µm

Figure 5.2. Irisin immunoreactivity was detected in the atrium and ventricle of heart and skeletal muscle of zebrafish. Irisin immunoreactivity was detected in atrial and ventricular cardiomyocytes of zebrafish **(a, b)** In skeletal muscle, irisin immunoreactivity (Catalog # ab131390, 1:3000, Abcam, Ramona, Massachusetts) was detected at the myofibril filament within the myotubule **(C)**. Preabsorption control of irisin was used as negative control **(d)**. Nuclei are stained blue (DAPI). Images were taken at 40X magnification and scale bar = 100 µm (and 0.5 µm for inset).

5.3.3. Irisin Downregulates PGC-1 alpha, Myostatin a and Myostatin b mRNA Expression, and Upregulates Troponin C mRNA Expression *in vitro*

Irisin (0.1 nM, 10 nM) downregulated relative mRNA expression of PGC-1 alpha (F value – 19.99; P value – 0.02) (**Figure 5.3 a**) when compared to saline treated controls in zebrafish heart. In contrast, irisin (10 nM) upregulated relative mRNA expression of troponin C in zebrafish skeletal muscle (F value – 23.3; P value- 0.04) (**Figure 5.3 b**), while irisin (10 nM) downregulated the relative mRNA expression of myostatin-a in zebrafish heart (P value – 0.025) and muscle (P value – 0.023) (**Figure 5.3 c**). No significant effect on troponin C relative mRNA expression was detected in zebrafish heart at 0.1 nM (P value – 0.7), and 10 nM (P value – 0.3), when compared to controls (**Figure 5.3 b**). Irisin (0.1 nM, 10 nM) downregulated relative mRNA expression of myostatin b (P value – 0.019) (**Figure 5.3 d**) when compared to controls in zebrafish heart.

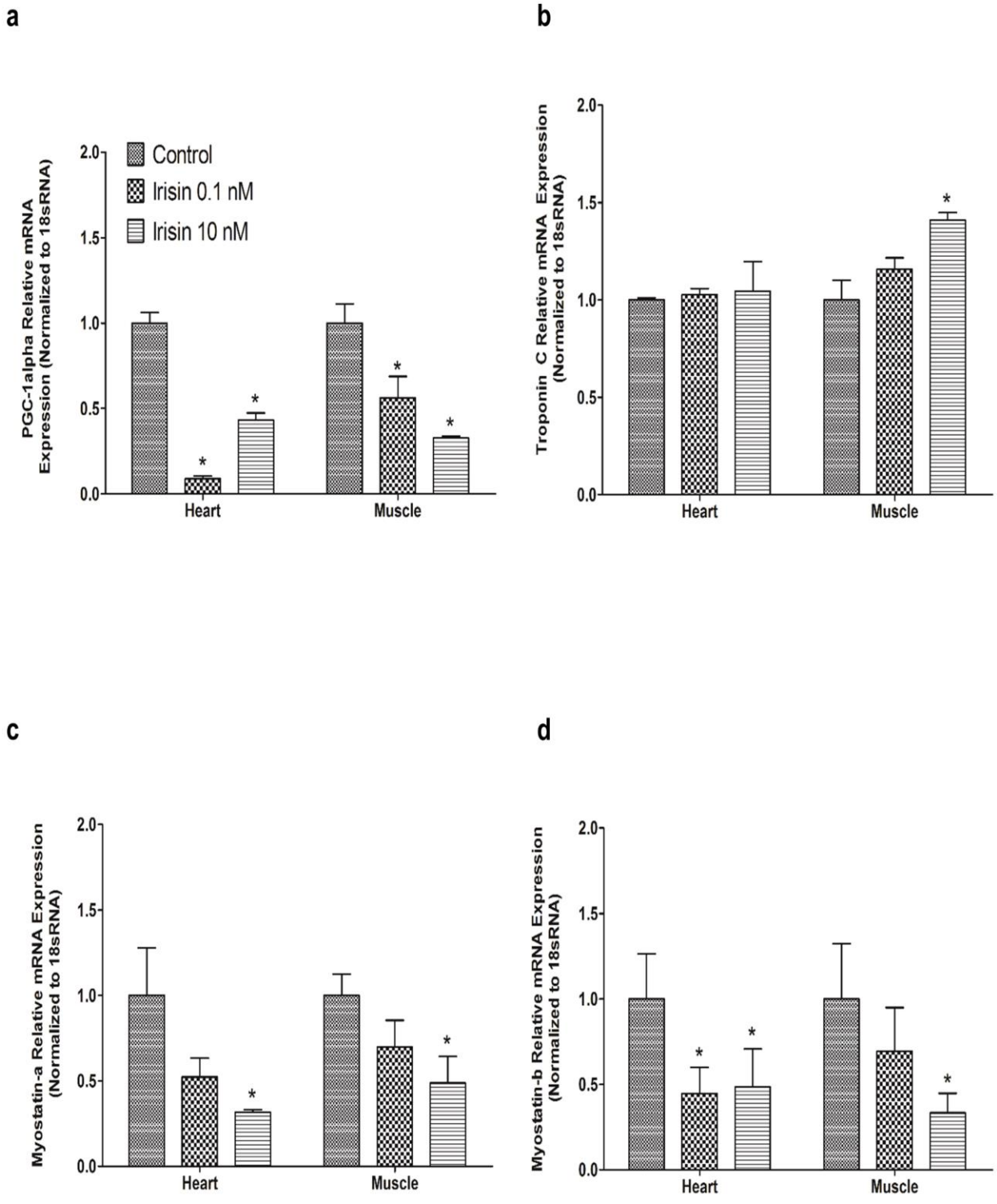

a
PGC-1alpha Relative mRNA Expression (Normalized to 18sRNA)
Control
Irisin 0.1 nM
Irisin 10 nM
2.0
1.5
1.0
0.5
0.0
*
Heart
Muscle
b
Troponin C Relative mRNA Expression (Normalized to 18sRNA)
c
Myostatin-a Relative mRNA Expression (Normalized to 18sRNA)
d
Myostatin-b Relative mRNA Expression (Normalized to 18sRNA)

Figure 5.3. Irisin downregulated mRNA expression of PGC-1 alpha, myostatin-a and b and upregulated troponin C mRNA expression in zebrafish. Groups include: Control; Irisin (0.1 and 10 nM). Heart and muscle were considered and the mRNA expression data was normalized to 18s RNA. Asterisks denote significant difference between the tissues (* $p<0.05$). Data is represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis.

5.3.4. Irisin Modulates Cardiac Function in Zebrafish

Representative long-axis brightness mode (B-mode) and color flow Doppler short axis views from the ultrasonography of adult zebrafish heart are shown in **Figures 5.4 A-C**. A single intraperitoneal injection of irisin (0.1 ng/g, 1 ng/g and 10 ng/g B.W) increased end-diastolic volume (F value – 51.32; P value – 0.032) in zebrafish (**Figure 5.5 a)**. Irisin (0.1 and 1 ng/g B.W) also increased heart rate (F value – 15.82; P value – 0.0254) and cardiac output (F value – 24.47; P value – 0.04) in zebrafish (**Figure 5.5 d, e**). siRNA enabled knockdown of irisin (10 ng/g B.W) significantly decreased end-diastolic volume (P value – 0.044), end-systolic volume (P value – 0.02), stroke volume (P value – 0.02), heart rate (P value – 0.038) and cardiac output (P value – 0.035) in zebrafish (**Figure 5.5 a-e)**. No significant effect on cardiac function was observed in response to 10 ng/g B.W irisin scrambled siRNA injection (P value – 0.09), when compared to control group (**Figure 5.5 a-e**).

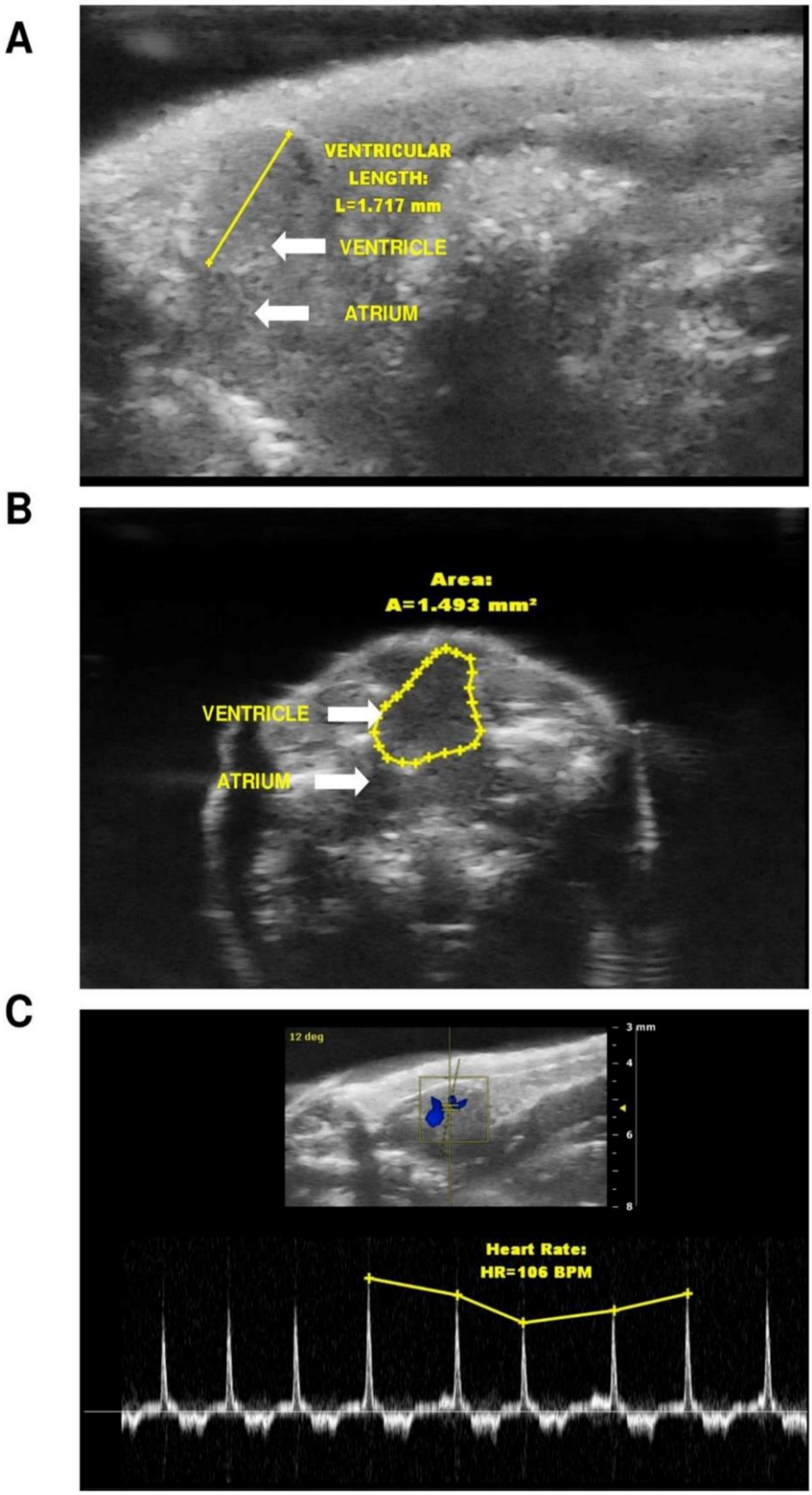

A
VENTRICULAR
LENGTH:
L=1.717 mm
VENTRICLE
ATRIUM
B
Area:
A=1.493 mm²
VENTRICLE
ATRIUM
C
12 deg
3 mm
4
6
8
Heart Rate:
HR=106 BPM

Figure 5.4. Ultrasound imaging of zebrafish heart. Representative long-axis (A), short axis (B) brightness mode (B-mode) view of adult zebrafish heart. Zebrafish were anesthetized, imaged and the long axis mode was considered to calculate the ventricular length (A), while short axis was considered to calculate ventricular area (B) (represented by blue line). Blood flow from atrium to ventricle through the atrioventricular valve are indicated by color (blue) in panel C using color flow Doppler mode and heart rate measurements were calculated by number of heart beats per 10 s during the B-mode ultrasound video loop, and converted to beats per minutes (bpm).

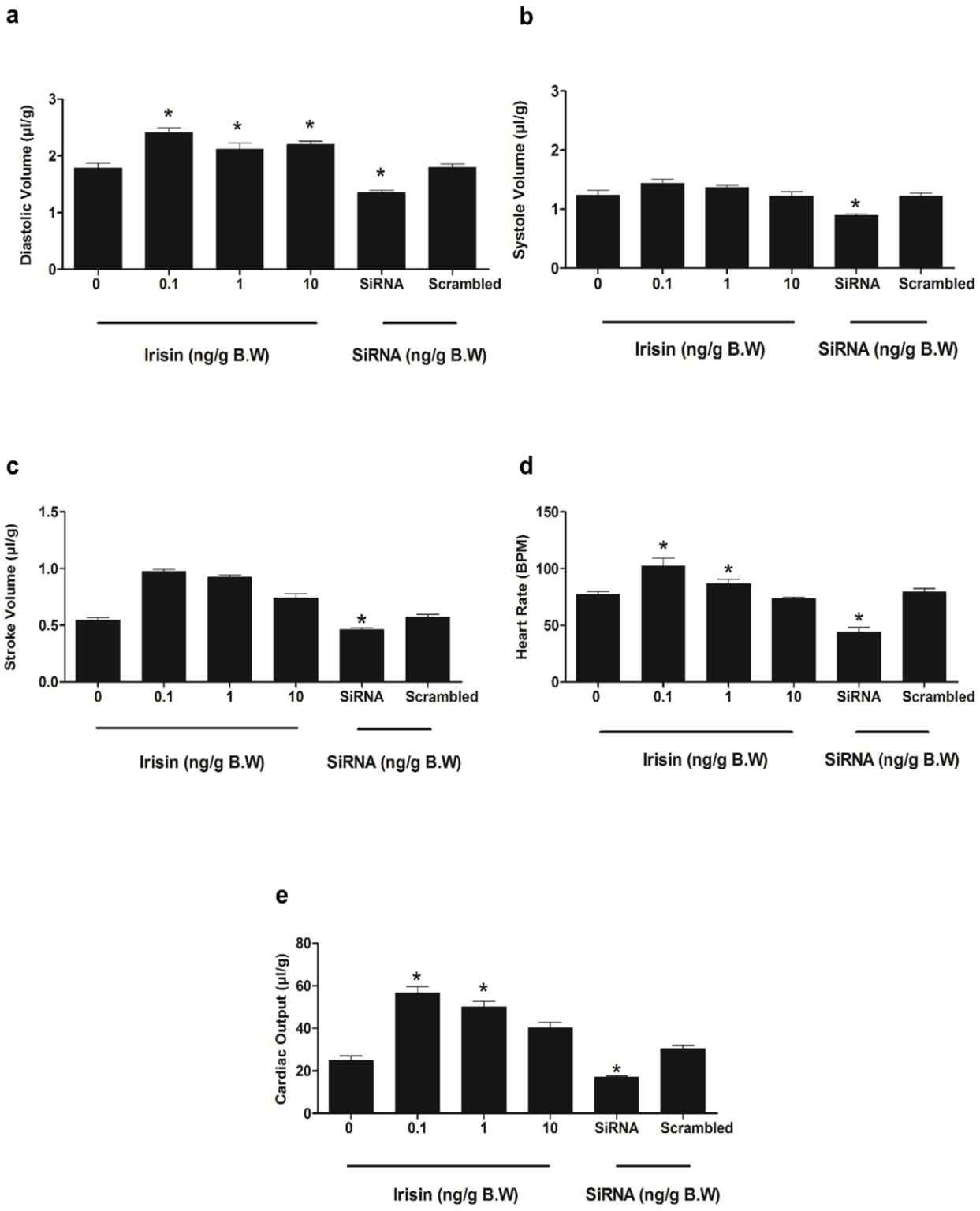

a
Diastolic Volume (µl/g)
3
2
1
0
*
*
*
*
0
0.1
1
10
SiRNA
Scrambled
Irisin (ng/g B.W)
SiRNA (ng/g B.W)
b
Systole Volume (µl/g)
3
2
1
0
*
0
0.1
1
10
SiRNA
Scrambled
Irisin (ng/g B.W)
SiRNA (ng/g B.W)
c
Stroke Volume (µl/g)
1.5
1.0
0.5
0.0
*
0
0.1
1
10
SiRNA
Scrambled
Irisin (ng/g B.W)
SiRNA (ng/g B.W)
d
Heart Rate (BPM)
150
100
50
0
*
*
*
0
0.1
1
10
SiRNA
Scrambled
Irisin (ng/g B.W)
SiRNA (ng/g B.W)
e
Cardiac Output (µl/g)
80
60
40
20
0
*
*
*
0
0.1
1
10
SiRNA
Scrambled
Irisin (ng/g B.W)
SiRNA (ng/g B.W)

Figure 5.5. Irisin administration enhances cardiovascular function, while knockdown of irisin by siRNA attenuated cardiac physiology in zebrafish. Groups include: Control; Dose of Irisin (0.1, 1, 10); Irisin siRNA (10 ng/g B.W); Scrambled siRNA (10 ng/g B.W). Units are displayed in the Y axis and different cardiac parameters were analyzed. Asterisks denote significant differences between control (saline group) and irisin, siRNA injected groups (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison test were used for statistical analysis.

5.3.5. Irisin Downregulates PGC-1 alpha, Myostatin a and b, and Upregulates Troponin C and Troponin T2D *In vivo* in Zebrafish

Exogenous administration of irisin (1 and 10 ng/g B.W) downregulated PGC-1 alpha mRNA expression (**Figure 5.6 a**) in zebrafish heart (F value – 22; P value – 0.024) and muscle (F value – 11.3; P value – 0.033) when compared to saline treated controls. On the other hand, exogenous irisin (0.1 and 1 ng/g B.W) upregulated troponin C (**Figure 5.6 b**) relative mRNA expression in zebrafish heart (P value – 0.027) and skeletal muscle (P value – 0.041). Intraperitoneal administration of 0.1 ng/g B.W, 1 ng/g B.W and 10 ng/g B.W downregulated myostatin a (**Figure 5.6 c**) relative mRNA expression in zebrafish heart (P value – 0.017) and skeletal muscle (P value – 0.041) when compared to saline treated group. Also, exogenous irisin (0.1 and 1 ng/g B.W) upregulated troponin T2D (**Figure 5.6 d**) relative mRNA expression in zebrafish heart (P value – 0.028) and skeletal muscle (P value – 0.032). Exogenous administration of irisin at 0.1 ng/g B.W, 1 ng/g B.W and 10 ng/g B.W downregulated myostatin b (**Figure 5.6 e**) relative mRNA expression in zebrafish heart (P value – 0.019) and skeletal muscle when compared to saline treated group. No significant effect was observed in response to an intraperitoneal injection of irisin (0.1 ng/g, 1 ng/g and 10 ng/g B.W) on beta-actin relative mRNA expression in zebrafish heart and skeletal muscle (**Figure 5.6 f**). Also, no effect in PGC-1 alpha (**Figure 5.6 a**) mRNA expression was observed after administration of 0.1 ng/g B.W of irisin in zebrafish heart and skeletal muscle.

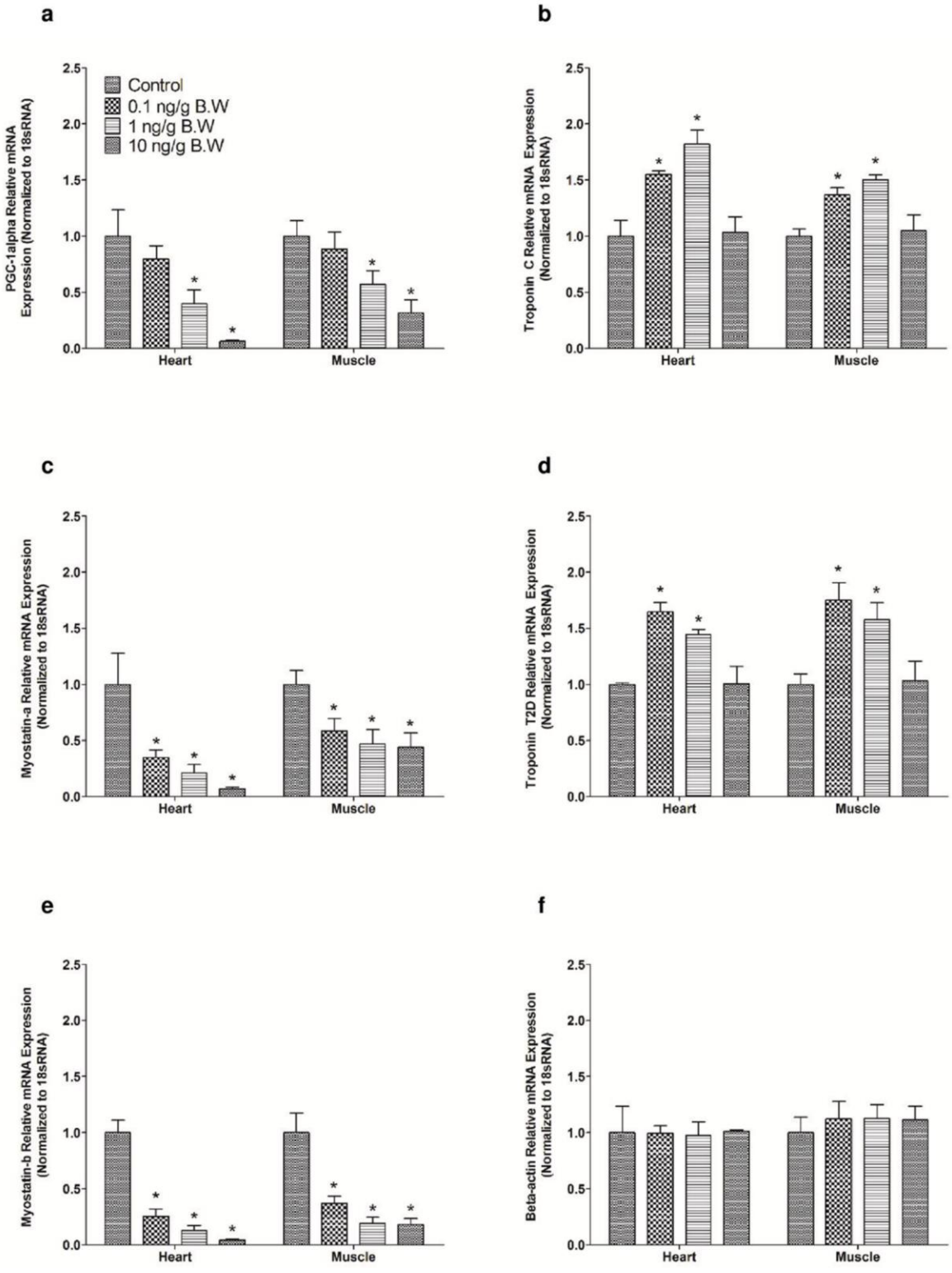

a
Control
0.1 ng/g B.W
1 ng/g B.W
10 ng/g B.W
PGC-1alpha Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
b
Troponin C Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
c
Myostatin-a Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
d
Troponin T2D Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
e
Myostatin-b Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
f
Beta-actin Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle

Figure 5.6. Exogenous irisin administration downregulated PGC-1 alpha, myostatin-a and b mRNA expression and upregulated troponin C and T2D mRNA expression in zebrafish. Exogenous irisin (1 ng/g and 10 ng/g B.W) significantly downregulated PGC-1 alpha **(a)** relative mRNA expression in zebrafish heart and skeletal Irisin (0.1 and 1 ng/g B.W) upregulated troponin C mRNA expression **(b)** in zebrafish heart and skeletal muscle when compared controls. However, exogenous irisin (0.1 ng/g 1 ng/g and 10 ng/g B.W) downregulated myostatin-a relative mRNA expression in zebrafish muscle and heart **(c)**. Upon exogenous administration of irisin at 0.1 ng/g and 1 ng/g B.W, troponin T2D relative mRNA expression was downregulated in zebrafish heart and skeletal muscle **(d)**. Also, irisin (0.1 ng/g, 1 ng/g and 10 ng/g B.W) downregulated myostatin-b relative mRNA expression in zebrafish when compared to saline treated controls **(e)**. No significant effect was observed in response to i.p injection of irisin towards beta-actin relative mRNA expression in heart and muscle of zebrafish **(f)**. Asterisks denote significant differences between control (saline group) and irisin, siRNA injected groups of the same (* $p<0.05$, n = 8 fish/group). Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison post hoc test were used for statistical analysis.

5.3.6. Knockdown of Irisin Upregulates Myostatin a and b, and Downregulates PGC-1 alpha, Troponin C and Troponin T2D in Zebrafish

Zebrafish irisin siRNA (10 ng/g B.W) downregulated PGC- 1 alpha (**Figure 5.7 a**) mRNA expression in zebrafish heart (F value – 16.34; P value – 0.032) and muscle (F value – 32.3; P value – 0.0254). *In vivo* (intraperitoneal) administration of zebrafish irisin siRNA (10 ng/g B.W) upregulated myostatin a and b (**Figure 5.7 b, c**) relative mRNA expression in zebrafish heart (F value – 24.47; P value – 0.031) and skeletal muscle (F value – 50.66; P value – 0.019), when compared to saline treated control group. Also, irisin siRNA downregulated troponin C (**Figure 5.7 d**) and troponin T2D (**Figure 5.7 e**) relative mRNA expression in heart (F value – 13.49; P value – 0.032) and skeletal muscle (F value – 25.65; P value – 0.041), when compared to saline treated control group. Injection of scrambled irisin siRNA (10 ng/g B.W) did not elicit any effects on myostatin a and b (P value – 0.7), PGC-1 alpha (P value – 0.09), troponin C (P value – 0.42) and troponin T2D (P value – 0.088) relative mRNA expression in zebrafish tissues (**Figure 5.7 a-e)**.

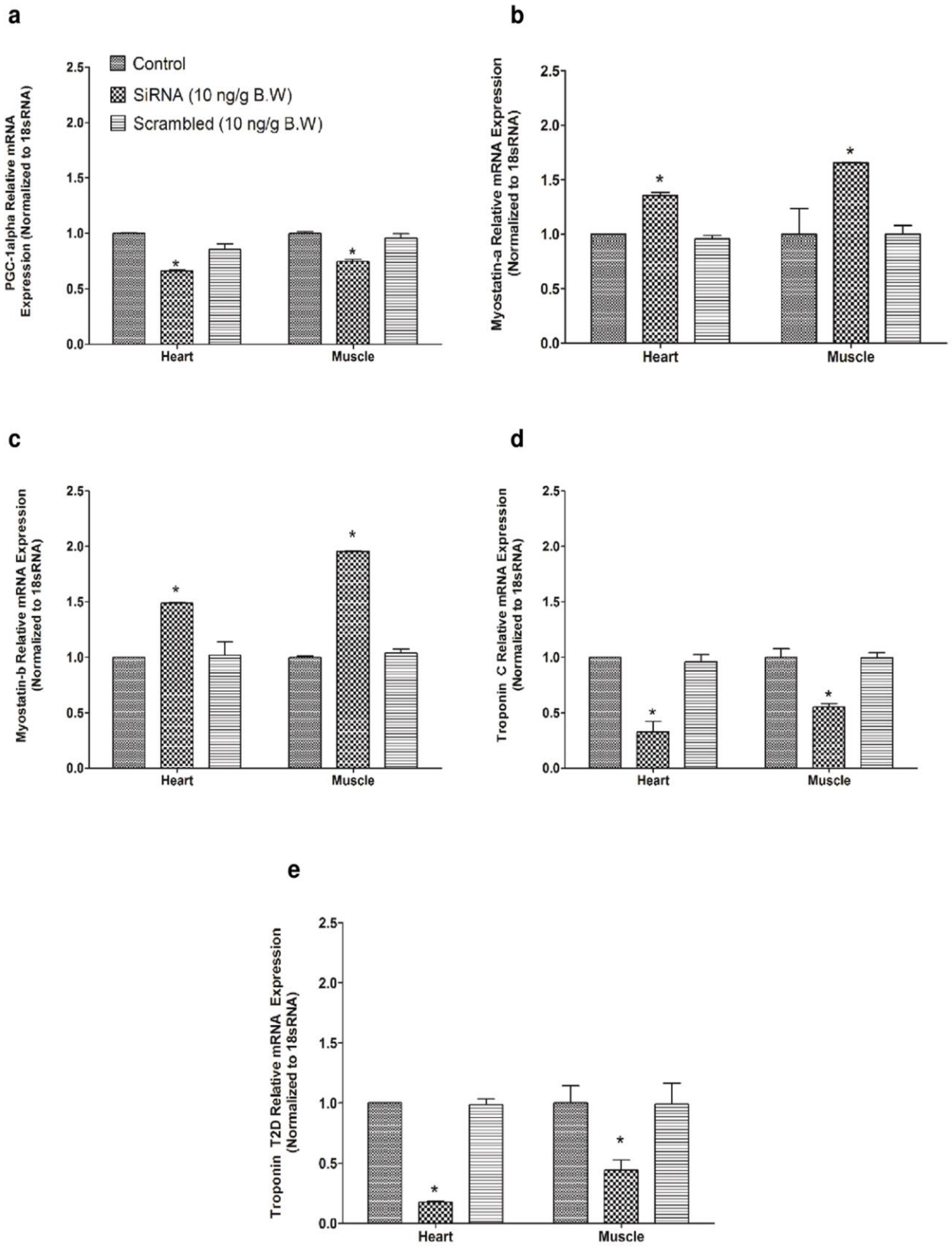

a
Control
SiRNA (10 ng/g B.W)
Scrambled (10 ng/g B.W)
PGC-1alpha Relative mRNA Expression (Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Heart
Muscle
b
Myostatin-a Relative mRNA Expression (Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Heart
Muscle
c
Myostatin-b Relative mRNA Expression (Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Heart
Muscle
d
Troponin C Relative mRNA Expression (Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Heart
Muscle
e
Troponin T2D Relative mRNA Expression (Normalized to 18sRNA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Heart
Muscle

Figure 5.7. Knockdown of irisin by siRNA altered the expression of mRNAs encoding cardiac muscle proteins in zebrafish. Groups include: Control; Irisin siRNA (10 ng/g B.W); Scrambled siRNA (10 ng/g B.W). Heart and skeletal muscle of zebrafish were considered and the relative mRNA expression of muscle proteins were normalized to 18s RNA. Data were represented as mean + SEM. One way ANOVA followed by Tukey's multiple comparison test were considered as statistical analysis.

5.4. Discussion

Irisin is a myokine derived from skeletal muscle in response to exercise, which has been shown to play a role in regulating cardiac function and angiogenesis in mice [39, 349]. Circulating irisin and adipose tissue FNDC5 were found to attenuate hyperglycemia, visceral adiposity and extramyocellular lipid deposition in obesity and type 2 diabetes in humans [233, 350]. In addition to this, lower levels of irisin was detected in circulation of individuals with gestational diabetes mellitus [351]. These results suggest a role for irisin in the maintenance of energy homeostasis in normal and disease conditions. In this research, we focused on cardiac expression of irisin and its role in cardiac physiology and muscle proteins in zebrafish. Irisin immunoreactivity was detected in zebrafish atrial and ventricular cardiomyocytes. Irisin immunoreactivity was detected in the myofibrils within the myotubules in skeletal muscle of zebrafish. These observations are in agreement with previous results that detected irisin immunoreactivity in the perimysium, endomysium and skeletal muscle nuclei in Sprague-Dawley rats, with a reported increase in cell specific expression upon exercise [237]. In addition to that, abundant irisin immunoreactivity was detected in the myocardium and connective tissues of heart in young rats. In the current study, the cell specific localization of irisin in skeletal and heart muscle suggests that irisin is a key promoter in regulating muscle proteins and cardiac function in zebrafish.

Our next study focused on determining whether irisin has any effects on cardiac physiology and modulation of muscle proteins in zebrafish. *In vivo* administration (i.p.) of irisin elicited an overall stimulatory effect on cardiac function in zebrafish. Intracerebroventricular injection of irisin increased cardiac output and blood pressure in rats

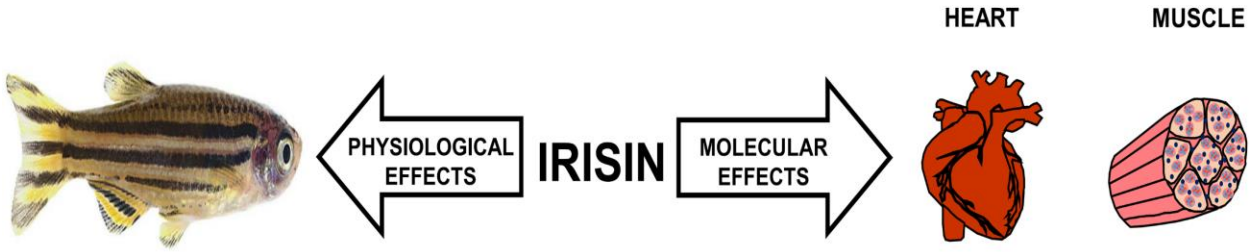
by activating hypothalamic paraventricular nuclei (PVN) neurons, while peripheral (intravenous) administration, or injection into the nucleus ambiguus decreased blood pressure, decreased cardiac function and caused vasodilation [22, 274]. A limitation in comparing these studies lies in the routes of administration (central *vs.* intravenous *vs.* intraperitoneal) chosen. The discrepancies in outcomes obtained in these studies could be due to variations in routes of administration, doses used, species-specificity, and mechanism of action in model organisms tested. Cardiac stimulatory effects of irisin are, at least in part, mediated by β-adrenoceptors and irisin-mediated sympathetic stimulation based on competitive effects with atenolol in rats [22]. However, vagal stimulation was instead suggested to mediate the previously reported decrease in cardiac function and blood pressure reported in rats injected with irisin in the nucleus ambiguous [274]. Overall, at the doses tested, using the i.p route of administration, irisin plays a stimulatory role on cardiac filling, contractility and heart rate in zebrafish. Some of these effects were found at the lower dose(s) tested, while at higher concentrations the responses were diminished or disappeared. One possibility is that at the higher doses of irisin tested, there might be receptor desensitization or downregulation, which results in the lack of responses found at lower doses. The mechanisms and pathways that mediate *in vivo* irisin actions on zebrafish cardiovascular biology warrant further studies.

In an attempt to understand possible mechanisms of irisin action, we then measured muscle proteins that were found to change in response to irisin in our *in vitro* studies. Our *in vitro* studies revealed that irisin (0.1 nM and 10 nM) downregulated PGC-1 alpha mRNA expression in zebrafish heart and muscle. Similarly, exogenous administration of irisin (1 ng/g and 10 ng/g B.W) reduced PGC- 1 alpha, and myostatin a and b relative mRNA expression in

zebrafish. This reduction in expression of PGC-1 alpha relative mRNA expression is in accordance with previous studies that found irisin secretion from skeletal muscles upon PGC-1 alpha activation during endurance training [39]. Knockdown of irisin upregulated myostatin a and b mRNA expression in zebrafish heart and skeletal muscle. Previous results have reported that increased expression of myostatin led to muscle wasting during aging in humans [352]. Overexpression of myostatin resulted in immobilization leading to muscle atrophy in mice [353]. Whether the overexpression of myostatin a and b could influence muscular development and muscle physiology in zebrafish needs further investigation. Administration of irisin resulted in significant increase in troponin C and troponin T2D mRNA in zebrafish skeletal muscle and heart, while knockdown of irisin downregulated troponin T2D and troponin C in heart and skeletal muscle of zebrafish. Troponin T2D plays an important role in sarcomere assembly and regulation of actin-myosin activity in zebrafish muscle [218, 354]. Morpholino knockdown of troponin T in zebrafish caused suppressive effect in pre-myofibril production leading to abnormalities in myofibrillogenesis [355]. Abnormality in troponin T expression was responsible for uncoupling myofibrillar calcium sensitivity in humans with hypertrophic obstructive cardiomyopathy [356]. Collectively, these results identify myostatin and troponin as novel targets of irisin. Although not a primary focus of this research, we found a decrease in beta tubulin in muscle collected from irisin siRNA treated zebrafish is that the protein samples in the siRNA lanes could have been degraded. Further research to elucidate the mediators and mechanism of cardiac action of irisin is warranted.

5.5. Conclusion

In conclusion, this research using approaches to add or remove irisin, indicate a primarily positive role for this peptide on zebrafish cardiac function. While some of our results are in agreement with similar studies in rodents, it appears that species-specific differences in irisin actions exist. We found muscle proteins as novel targets of irisin, and this suggest a modulatory role for these proteins in regulating cardiac function. **Figure 5.8** summarizes the effects of irisin on cardiovascular physiology and regulation of muscle proteins in zebrafish. Our results establish irisin as a potent bioactive molecule zebrafish. It also provides the basis for future research on the mechanisms of action of irisin, and its role in other physiological processes in zebrafish.



	Irisin	Irisin siRNA
Diastolic Volume	↑	↓
Systole Volume	↔	↓
Stroke Volume	↔	↓
Heart Rate	↑	↓
Cardiac Output	↑	↓

	Irisin	Irisin siRNA
PGC-1α	↓	↓
Troponin C, T2D	↑	↓
Myostatin a,b	↓	↑

↑= Stimulatory effect; ↓ = Inhibitory effect; ↔ = No effects.

Figure 5.8. Summary of irisin effect on cardiac function and regulation of muscular proteins in zebrafish. Scheme depicting the role of irisin on cardiovascular physiology, and in the regulation of muscle proteins in zebrafish. Irisin is abundantly expressed in cardiac and skeletal muscles, and has a positive role on cardiac functions (indicated by upward arrow), and muscular proteins in zebrafish. Knockdown of irisin played an important role in modulating muscle proteins mRNA and cardiovascular physiology in zebrafish (indicated by down arrow). Together, irisin is a positive modulator towards cardiac physiology and regulates muscular proteins in zebrafish.

TRANSITION

Chapters 4 and 5 focused on the role of irisin effects on feeding and regulating cardiovascular functions, and the modulation of the mRNAs of the appetite regulatory proteins and muscle proteins in zebrafish. Together, these results provide support for the appetite regulatory and cardiac functions in zebrafish. In order to determine the potential mechanisms of irisin effects on cardiovascular biology, in the research described in the next chapter, we explored whether adrenergic pathways mediate the cardiovascular effects of irisin.

Publication: Sundarrajan L, Jithine JR, Weber LP, Unniappan S., 2018. Irisin Follows an Adrenergic Pathway to Regulate Cardiovascular Functions in Zebrafish. *Manuscript in Preparation.*

Contributions: The project was completed in collaboration with Dr. Lynn Weber, who provided guidance in experimental design and data analysis and interpretation, and shared the ultrasound equipment to conduct studies in zebrafish. Sundarrajan planned and executed all studies, analyzed data, and prepared the manuscript draft. Jithine JR helped in conducting ultrasound studies. Dr. Suraj Unniappan provided the original ideas and funding. Drs. Weber and Unniappan helped design experiments and assisted with *in vivo* studies, tissue sampling, data analysis and interpretation, and manuscript preparation.

Chapter 6

Irisin Regulation of Cardiac Functions in Zebrafish: A Role for the Autonomic Nervous System?

6.1. Introduction

Skeletal muscle contributes up to 40% of the total body weight mass and is considered an exercise dependent endocrine organ [336, 337]. Skeletal muscle also secretes cytokines and myokines that exhibit autocrine and paracrine effects in humans [357]. Irisin is a recently discovered exercise-induced, 23 kDa myokine abundantly expressed in the cardiac and skeletal muscles of mammals [358]. It is encoded in FNDC5, a 212- amino acid precursor. FNDC5 undergoes post-translational processing in skeletal muscle to produce irisin, which is then released into circulation [359]. Circulating levels of irisin are ~3.6 ng/mL in resting individuals, while it is ~4.3 ng/mL in individuals undergoing exercise [46]. The activation of FNDC5 is regulated by PGC-1α, which stimulates the synthesis and secretion of irisin into circulation [39, 46]. In humans, the expression of FNDC5 mRNA is highly concentrated in the skeletal muscle, brain, adipose tissue, gut and pericardium [39, 272, 273, 360]. FNDC5 in adipocytes reduced UCP1 expression and stimulated adipogenesis [358]. FNDC5 suppressed adipose tissue inflammation and insulin resistance via AMPK mediated pathway [243]. Increased levels of irisin stimulated the expression of thermogenin in white adipose cells [236]. In rodents, irisin immunoreactivity (IR) was localized in skeletal and cardiac muscles, as well as the Purkinje cells in the cerebellum and neuroglial cells [234, 237, 238]. Overexpression of irisin in obese mice resulted in increased energy expenditure, decreased body weight, improved glucose homeostasis and decreased insulin resistance [39, 275, 276]. In zebrafish, administration of irisin promoted

angiogenesis and modulated matrix metalloproteinase activity via ERK signaling pathway [47]. My research outlined in **chapter 4** described the role of endogenous irisin in regulating food intake and the expression of mRNAs encoding appetite regulatory peptides [361]. In **chapter 5**, we also reported a major role for irisin in regulating cardiovascular functions and muscle proteins in zebrafish [306]. Our previous results showed that intraperitoneal injection of irisin (0.1 and 1 ng/g B.W) increased diastolic volume, heart rate and cardiac output, while siRNA based knockdown of endogenous irisin (10 ng/g B.W) resulted in opposite effects on cardiovascular function in zebrafish. Collectively, these results state that irisin modulates cardiac physiology in zebrafish, but the mechanism by which irisin regulates cardiac functions in fish remain poorly understood. Is the autonomic nervous system a mediator of irisin functions on cardiac tissue?

In vertebrates, the cardiac contraction, control of vascular resistance is mediated by the input from the autonomic nervous system (ANS; parasympathetic and sympathetic) [128, 129]. The ANS is comprised of sympathetic and parasympathetic systems [130, 131]. The sympathetic nervous system is initiated when body receives signals for emergency responses (flight or fight response). The cardiac sympathetic nervous system is mediated in the ANS when body undergoes stress [27, 130]. The preganglionic neurons arising from the sympathetic nervous system is localized at the upper thoracolumbar region of the spinal cord. The preganglionic fibres exit the spinal nerves through white rami branches followed by entry into the sympathetic ganglia. The cardiac neurons form the sympathetic ganglia are located along the visceral column (paravertebral ganglia with projecting into the postganglionic neurons).

The cardiac sympathetic nervous system is an important component of the autonomic nervous system that is responsible for the fight or flight response, whereas the parasympathetic nervous system controls the basic functions of heart [27]. The activation of sympathetic nervous system is enabled by two types of adrenergic receptors in the cardiovascular system: α (α_1 and α_2) and β (β_1 and β_2). In humans, the presence of α_1 and α_2 receptors are detected in the vascular smooth muscle and its activation leads to vasoconstriction. Beta - receptors are reported to have a functional role in the sympathetic portion of the autonomic nervous system. They are classified into two major subgroups as β1 or β2 receptors. β1 receptors are abundantly expressed in the cardiac muscle and ventricular cardiomyocytes of humans. The activation of β1 receptors increased heart rate, stimulated contractility and intracellular calcium signaling. In addition to this, β1 receptors were reported to have a key role in releasing renin from kidneys to help maintain blood pressure and plasma sodium level. In humans, β2 receptors have been detected in the skin, vascular and bronchial smooth muscle. The activation of β2 receptors has been shown to have a positive effect on vasodilatation, leading to decreased blood pressure [3]. Over the last decade, several adrenergic receptors have been discovered and various attempts were made to elucidate their physiological roles and mechanism of action [172, 362-364].

The parasympathetic nervous system is generally responsible for the basal organ system function. The regulation of parasympathetic nervous system plays an antagonistic role in cardiac function [27]. The preganglionic neurons associated with the parasympathetic nervous system is expressed from the neurons located in the mid brain region and medulla oblongata [27, 132, 133]. The preganglionic fibers innervate the organs of the thorax and upper abdomen, which carries around 75% parasympathetic nerve fibres passing to the heart and other peripheral

organs. These fibers synapse with the ganglion, and then reach the target organs via short postganglionic fibers [27, 128]. The predominant neurotransmitter of the parasympathetic nervous system is acetylcholine (ACh). ACh bind to two types of receptors namely, nicotinic and muscarinic receptors. Nicotinic receptors are expressed between the pre and post ganglionic synapses of the sympathetic and parasympathetic nervous system pathways [27, 132]. Neuromuscular junctions located between the skeletal muscle fibers are nicotinic and elicits rapid, excitatory response upon activation. In addition to that, muscarinic acetylcholine receptors (mAChRs) belong to the family of G-protein coupled receptors (GPCRs) that regulate numerous fundamental functions of central and peripheral nervous system in activating autonomous nervous system [365]. The mAchRs are classified into five distinct subtypes, M_1 to M_5 encoded by the genes CHRM1 to CHRM5. Among the subtypes, M_1, M_3 and M_5 have been shown to couple with G-proteins of $G_{q/11}$ family while M_2 and M_4 regulate through $G_{i/o}$ family of G proteins [365]. The mAchRs also play an important role in regulating heart rate, smooth muscle contraction and transport of Ca2+ ions from the sarcoplasmic reticulum [365]. In zebrafish, loss of M_2 muscarinic receptor resulted in a cardioinhibitory role for the ß- adrenergic receptor and inhibited the development of hypoxic bradycardia thereby resulting in increasing heart rate in fish [366]. In contrast to the functions of sympathetic nervous system, parasympathetic nervous system has moderate effect on cardiac function. The parasympathetic system effects on cardiac function and contractility are negative chronotropic effect (lower heart rate), negative inotropic effect (reduced myocardial contractility) and negative dromotropic effect (decreased conduction velocity) [27]. While majority of these are reported in mammals, limited information is also available in fish [132, 171, 172, 174, 175, 362].

The list of the autonomic antagonists is quite long [132, 171, 180, 362]. This discussion restricts to propranolol hydrochloride (beta blocker) and atropine sulfate (muscarinic antagonist), two autonomic nervous system blockers used in this thesis research. Propranolol, an adrenergic antagonist is widely used to treat high blood pressure, cardiac arrhythmias and angina pectoris [174]. Propranolol has been reported to suppress the sympathetic nervous system by lowering blood pressure, reducing heart rate and heart contractility [363, 367, 368]. The mechanism of action of propranolol involves inhibition of adrenergic stimuli by blocking the β-adrenergic receptor (β_1) within the myocardium. By blocking β-adrenergic receptors, propranolol lowers heart rate, and decreases cardiac output and contractility [172, 174, 367, 369, 370]. Exposure to propranolol (0.1 µg/L) decreased heart rate and impaired cardiac function after 44 h post fertilization in zebrafish [187]. Atropine sulfate, a muscarinic receptor antagonist is another drug widely used to improve heart rate and offer adequate respiratory support for patients to support cardiac function and maintain blood pressure [371]. Atropine increased heart rate by blocking the muscarinic receptors at the cardiac sinoatrial node causing tachycardia in mammals [313, 372]. Administration of atropine caused a significant increase in heart rate in healthy dogs [373]. The mechanism of action of atropine sulfate involves inhibition of muscarinic receptors and inhibit the action of the vagus nerve on the heart [374]. This inhibition leads to the blockade of the normal tonic firing of parasympathetic nervous system found in most species, resulting in increased cardiac output and heart rate [27, 375-377].

Is sympathetic nervous system a mediator of cardiac effect of exogenously administered irisin and conversely, is the parasympathetic system a mediator of the cardiac effects of irisin siRNA? If it is, can irisin modulate cardiac function in the presence of a beta blocker? In order to

address these, adrenergic receptor (propranolol) and muscarinic receptor blockers (atropine) were used either separately, or co-injected with irisin or irisin siRNA in zebrafish. We hypothesized that the adrenergic system mediates irisin regulation of cardiovascular functions in zebrafish. Ultrasound monitoring of *in vivo* cardiac functions, and measurement of mRNAs encoding cardiac proteins were conducted. Our results indicate adrenergic mediation of cardiovascular functions of exogenously administered irisin in zebrafish.

6.2. Materials and Methods

6.2.1. Animals

Male and female zebrafish (*Danio rerio*; 3-4 months old; body weight ~1.5 g) were purchased from the Aquatic Toxicology Centre, University of Saskatchewan. Zebrafish were housed and maintained at 27° C under 12L: 12D photoperiod cycle. Fish were fed once a day (10:00 AM) with 5% body weight slow sinking pellets (Aqueon, Catalog # 06053). Unless otherwise specified, fish were anesthetized using 0.5% TMS-222 followed by spinal transection. All animal studies adhered to the policies of the Canadian Council for Animal Care and were approved by the University of Saskatchewan Animal Research Ethics Board (2012-0033).

6.2.2. Is Irisin a Modulator of Cardiac Function in the Presence of Beta Blocker?

Cardiac parameters were measured using a VEVO 3100 high frequency Machine (Visual Sonics, Markham, ON) equipped with B-mode imaging and pulsed-wave Doppler as described earlier [21, 122]. Zebrafish (n=6/group) were anesthetized using Aquacalm (20 mg/L, Syndel Laboratories, Canada), and were intraperitoneally injected with 6 µL of 0.1 ng/g B.W synthetic human irisin (Catalog # 067-16; Phoenix Pharmaceuticals, Burlingame, CA) alone, or with 100

ng/g B.W propranolol (P-0884; 1G; Sigma Aldrich, Germany). The effective doses for irisin was determined in our previous studies (**Chapter 5**), and for propranolol, from an already reported cardiovascular study in zebrafish by Finn et al., [187]. Control fish (n=6) were injected with saline (Catalog # JB1323, 0.9% sodium chloride; Baxter Corporation, IL, USA). Post-intraperitoneal injections, zebrafish were allowed to recover and ultrasound measurements were recorded at 15 min post-injection. For cardiac measurements, fish were transferred to a Styrofoam-lined holding dish and placed ventral side up with recirculating water containing Aquacalm (20 mg/L) maintained at 27±0.5 °C to maintain anesthesia throughout the ultrasound analysis, and minimal impact towards cardiovascular functions in zebrafish [21, 122].

A MX700 scan head was used to obtain short and long axis views of zebrafish ventricle using B-mode. The three different short axis areas along the ventricular side were measured as A1, A2, A3 while the long axis ventricular length was measured and divided by three to give the ventricular height (h) as per Eq (1). All values under the equation were measured at both systole and diastolic volumes (mm^3= µl) using Visual sonics software (Markham, ON). The end systolic and diastolic volumes for each ventricle of zebrafish were measured using this equation:

$$V= (A1+A2)\ h + ((A3h)/2) + (\pi/ 6\ (h^3)$$

To calculate the stroke volume, end systolic volume was subtracted from end diastolic volume. Heart rate was calculated by counting the number of heart beats per 10 s during the B-mode ultrasound video loop and converted to beats per minute (bpm). Cardiac output was determined by multiplying stroke volume (SV) and heart rate (bpm).

Cardiac output= Heart rate (bpm) * Stroke volume (SV)

The body weight of each fish was noted, and the output data were normalized to body weight and analyzed further using statistical analysis.

6.2.3. Knockdown of Irisin Using siRNA in the Presence of Atropine – Role in Cardiovascular Functions?

Zebrafish irisin siRNA was custom synthesized (Table 6.1; Dharmacon, Montreal, CA). Scrambled sequences of irisin siRNA were designed to test whether any siRNA sequences that shares similar length, but highly dissimilar orientation of the sequence could elicit same biological activity in zebrafish. Scrambled siRNA sequences of irisin were designed using GenScript sequence scramble tool (https://www.genscript.com/ssl-bin/app/scramble) from zebrafish siRNA sequence, and synthesized by Dharmacon (**Table 6.1**). Zebrafish were intraperitoneally injected with either irisin siRNA (10ng/g B.W) or scrambled siRNA (10 ng/g B.W) alone or in combination with atropine (100 ng/g B.W) (Catalog # A0132-5G; Sigma Aldrich, Germany). Post-injection, zebrafish were allowed to recover for a period of 15 min, and transferred for ultrasound monitoring as described earlier (***Chapter 6.2.2***). Stroke volume, cardiac output, diastolic volume was calculated according to equation 1. The body weights of fish were measured, and the output data were normalized to body weight and analyzed further using statistical analysis.

Table 6.1. Forward and reverse sequence of irisin and scrambled siRNA used during gene knockdown studies.

siRNA	siRNA sequence (5'-3')		Length
	Forward	Reverse	
Irisin	C.C.A.A.A.G. A.G.U.C.A.G. A.G.A.A.A.C. U.U.U	P.A.G.U.U.U.C.U.C.U.G.A.C.U. C.U.U.U.G.G.U.U	21
Scrambled	G.C.G.U.A.U. C.A.A.C.G.G. A.G.U.U.A.U. A.U.U	P.U.A.U.A.A.C.U.C.C.G.U.U.G. A.U.A.C.G.C.U.U	21

6.2.4. Irisin in the Presence of Propranolol – Does it Affect Muscle Proteins in Zebrafish?

Following ultrasound monitoring and post 1 hour injection, heart and skeletal muscle were collected from all groups following zebrafish euthanasia using TMS-222 and used for studying the effects of irisin on the expression of mRNAs encoding muscle proteins in the presence of propranolol. Briefly, total RNA was extracted using TRIzol method as described in Chapter 3.3.3.1. cDNA synthesis and RT-qPCR were conducted as described in section **2.2.3**. Data were normalized to 18s RNA (housekeeping gene) (**Table 6.2**).

Table 6.2. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)	Gene Accession Number
	Forward	Reverse		
PGC- 1 alpha	TCTATTCG GAAGGGCCCAGA	GGTGGTGCTG TCTCGTTTTG	58	XM_017357139.2
Myostatin-b	TCCTTTAGC ACGCCTTGGAA	TGCTTGAGTC GGAGTTTGCT	60	NM_131019.5
Myostatin-a	TTTTGAGCA TCCTGCGCCTA	ATCTTTGGGCT CAGTGCGAA	60	NM_001004122.2
Troponin-C	GCAGAAAAA TGAGTTCCGTGC	TTCCGCCAGT TCTTCCTCTG	60	AF180890.1
Troponin T2D	AGTTCAGGAGG AAGTGGATGAGT	AGTCTGGCTT GACGCTCTTTC	60	NM_001025179.1
18s	GGATGCCCTTA ACTGGGTGT	CTAGCGGCGC AATACGAATG	60	KY486501.1

6.2.5. Irisin siRNA in the Presence of Atropine – Regulation of Muscle Proteins in Zebrafish?

Following ultrasound monitoring, heart and muscle were collected from all groups and stored at –80° C for total RNA extraction and cDNA extraction as described in **2.2.3**. Sampling was done at approximately 1-hour post ultrasound monitoring. In order to determine the effect of irisin siRNA and scrambled siRNA on the mRNAs encoding the muscle proteins in the presence of atropine, RT-qPCR were carried out using troponin C, PGC-1 alpha, beta-actin, myostatin-a and myostatin-b and normalized to 18s RNA (housekeeping gene) (**Table 6.2**).

6.2.6. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test using PRISM version 5 (GraphPad Inc., USA) and IBM SPSS™ version 21 (IBM, USA) were used for statistical analysis. $P < 0.05$ was considered statistically significant. Data are represented as mean + SEM. For ultrasound data analysis, one-way analysis of variance (ANOVA) and t-test were used, and data were normalized to the body weight of fish.

6.3. Results

6.3.1. Propranolol Alone Reduced, and in Combination with Irisin, Attenuated Effects of Irisin on Cardiovascular Parameters in Zebrafish

A single I.P. injection of irisin (0.1 ng/g B.W) increased the end-diastolic volume (F value – 20.8; P value – 0.033) in zebrafish (**Figure 6.1 A**). Irisin (0.1 ng/g B.W) also increased heart rate (F value – 28.3; P value – 0.032) and cardiac output (F value – 44.4; P value – 0.041) in zebrafish (**Figure 6.1 D and E**) when compared to saline injected control group. I.P. administration of propranolol (100 ng/g B.W) alone decreased end diastolic (P value – 0.022) and systolic volume (P value – 0.029), stroke volume (P value – 0.035), heart rate (P value – 0.028), and cardiac output (P value – 0.031) in zebrafish (**Figure 6.1 A-E**). Irisin (0.1 ng/g B.W) effects on end diastolic (P value – 0.018) and end systolic volume (P value – 0.026), and stroke volume (P value – 0.041) were attenuated in the presence of propranolol (**Figure 6.1 A-C**). Irisin was ineffective in modulating heart rate (P value – 0.87) and cardiac output (P value – 0.23) in zebrafish in the presence of propranolol (**Figure 6.1 D and E**)

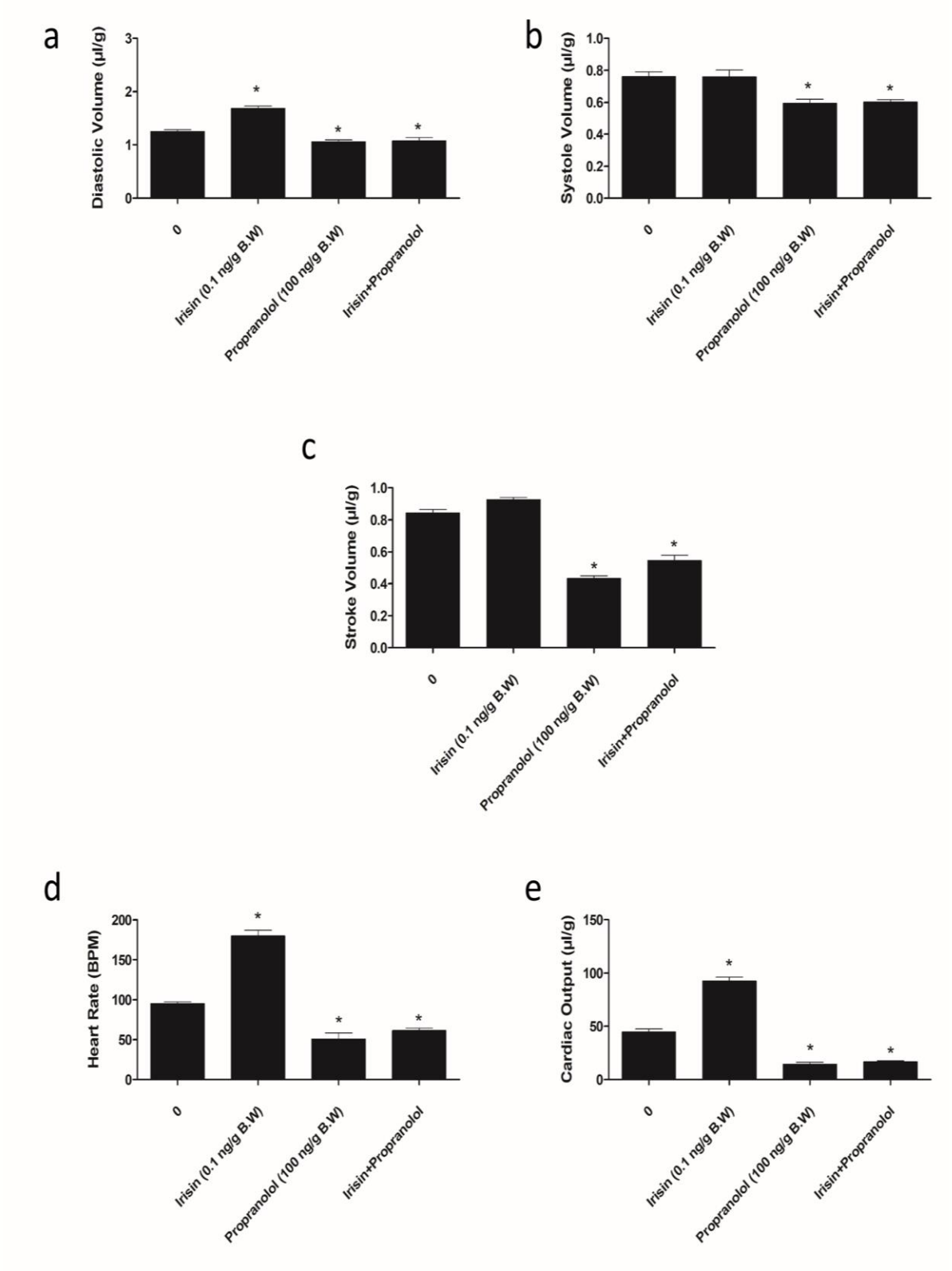

a
Diastolic Volume (µl/g)
3
2
1
0
*
*
*
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol
b
Systole Volume (µl/g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol
c
Stroke Volume (µl/g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol
d
Heart Rate (BPM)
200
150
100
50
0
*
*
*
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol
e
Cardiac Output (µl/g)
150
100
50
0
*
*
*
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol

Figure 6.1. Administration of irisin increased cardiovascular functions; while propranolol decreased cardiac function in zebrafish. Groups include: Control, Irisin (0.1 ng/g B.W), Propranolol (100 ng/g B.W), Irisin + Propranolol. Cardiac parameters were determined by ultrasound imaging and the units are displayed in Y axis. Asterisks denote significant differences between control group and irisin group and irisin co-injected with propranolol group. Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison tests were used for statistical analysis (*P<0.05 (Compared to control, N=6 fish/group).

6.3.2. Atropine Increased Cardiac Function, while it was Ineffective in Modulating Irisin Effects on Cardiac Functions in Zebrafish

siRNA knockdown of irisin (10 ng/g B.W) decreased end-diastolic (F value – 16.66; P value – 0.041) and systolic volumes (F value – 28.23; P value – 0.032), stroke volume (F value – 15.82; P value – 0.0112), heart rate (F value – 22.27; P value – 0.04) and cardiac output (F value – 51.31; P value – 0.039) in zebrafish (**Figure 6.2 A-E**) when compared to saline injected controls. I.P injection of atropine (100 ng/g B.W) alone only significantly increased heart rate (P value – 0.043) which led to a significant increase in cardiac output (P value – 0.018), while atropine co-injected with irisin siRNA was ineffective in modulating cardiac functions (P value – 0.36) in zebrafish (**Figure 6.2 A-E**), when compared to irisin siRNA-injected group. In other words, atropine showed to ability to reverse cardiac effects of irisin siRNA.

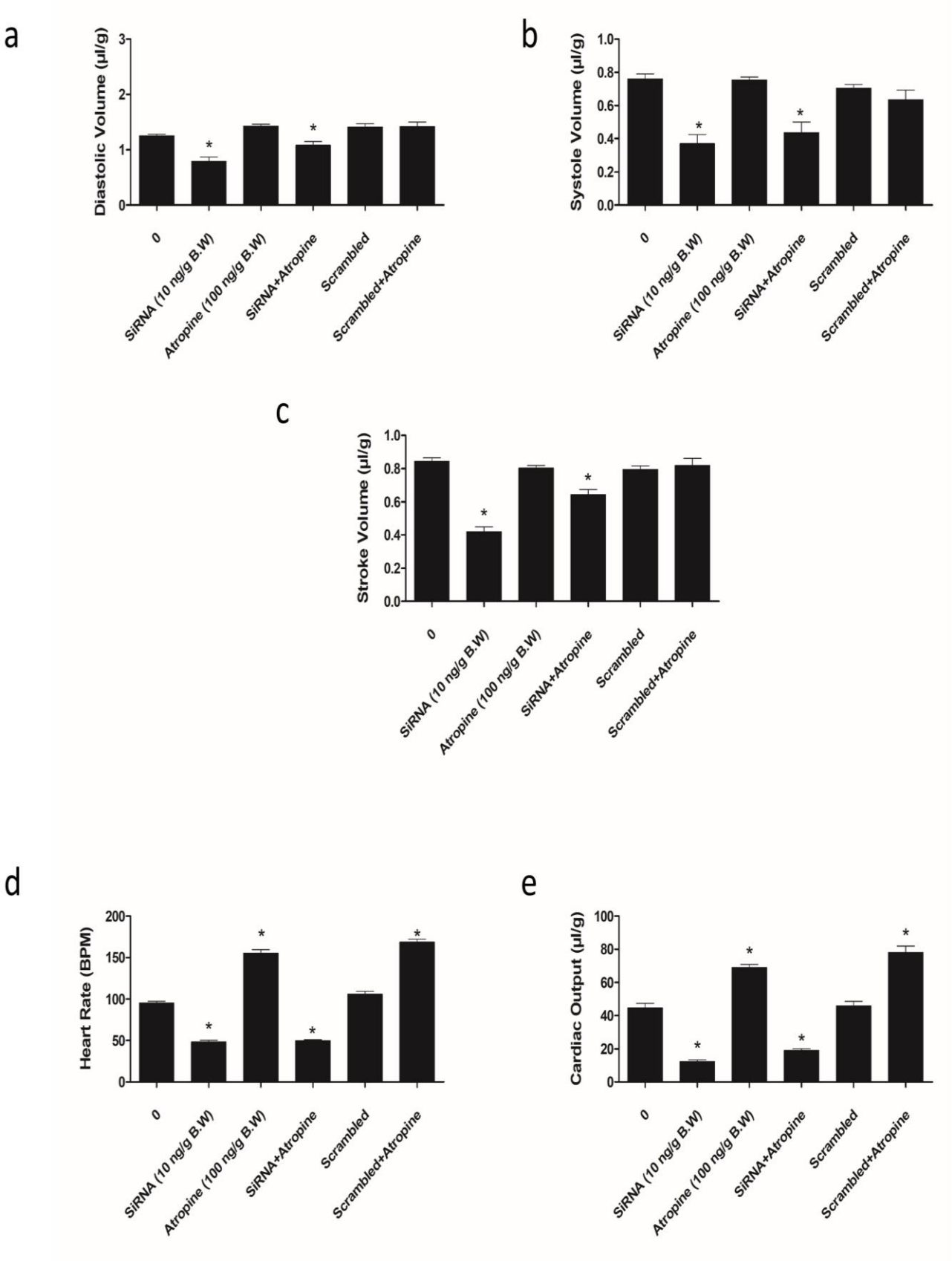

a
Diastolic Volume (µl/g)
3
2
1
0
*
*
0
SiRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled
Scrambled+Atropine
b
Systole Volume (µl/g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
SiRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled
Scrambled+Atropine
c
Stroke Volume (µl/g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
SiRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled
Scrambled+Atropine
d
Heart Rate (BPM)
200
150
100
50
0
*
*
*
*
0
SiRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled
Scrambled+Atropine
e
Cardiac Output (µl/g)
100
80
60
40
20
0
*
*
*
*
0
SiRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled
Scrambled+Atropine

Figure 6.2. Knockdown of irisin by siRNA attenuated cardiac function, while atropine modulated cardiac function in zebrafish. Groups include: Control, Irisin siRNA (10 ng/g B.W), Atropine (100 ng/g B.W), Irisin siRNA + Atropine, Scrambled siRNA (10 ng/g B.W) and Scrambled siRNA + Atropine. Cardiac parameters were determined by ultrasound imaging and the units are displayed in Y axis. Asterisks denote significant differences between control group, irisin siRNA group and irisin siRNA co-injected with atropine group. Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison tests were used for statistical analysis (*P<0.05 (Compared to control).

6.3.3. Adrenergic Pathway Mediates Irisin Regulation of Muscle Proteins in Zebrafish

Irisin (0.1 ng/g B.W) downregulated PGC-1 alpha mRNA, myostatin-a and myostatin-b mRNA expression in zebrafish heart (F value – 28.23; P value – 0.038) and muscle (F value – 32.3; P value – 0.022) (**Figure 6.3 A, C and E**). Administration of irisin (0.1 ng/g B.W) upregulated troponin C and troponin T2D mRNA expression in zebrafish heart (F value – 23.23; P value – 0.024) and skeletal muscle (F value – 47.9; P value – 0.014) tissues (**Figure 6.3 B and D**). Propranolol (100 ng/g B.W) either alone or in the presence of irisin (0.1 ng/g B.W) had similar effects on the regulation of mRNAs encoding muscle proteins when compared to irisin siRNA effects in zebrafish (P value – 0.023) (**Figure 6.3 A-E**). On the other hand, zebrafish irisin siRNA (10 ng/g B.W) downregulated PGC-1 alpha, troponin C and troponin T2D mRNA expression in zebrafish heart (P value – 0.033) and skeletal muscle (P value – 0.026) (**Figure 6.4 A, B and D**). Irisin siRNA (10 ng/g B.W) downregulated myostatins a and b mRNA expression in heart (P value – 0.0119) and skeletal muscle (P value – 0.031) tissues of zebrafish (**Figure 6.4 C and E**). Atropine (100 ng/g B.W) either alone or in the presence of irisin siRNA (0.1 ng/g B.W) had similar effects on regulation of mRNAs of the muscle proteins when compared to irisin siRNA effects in zebrafish (P value – 0.042) (**Figure 6.4 A-E**). Injection of scrambled irisin siRNA either alone or in the presence of atropine did not elicit any changes on myostatin-a (P value – 0.81) and b (P value – 0.22), PGC-1 alpha (P value – 0.96), troponin C (P value – 0.089) and troponin T2D (P value – 1.1) relative mRNA expression in zebrafish tissues (**Figure 6.4 A-E**)

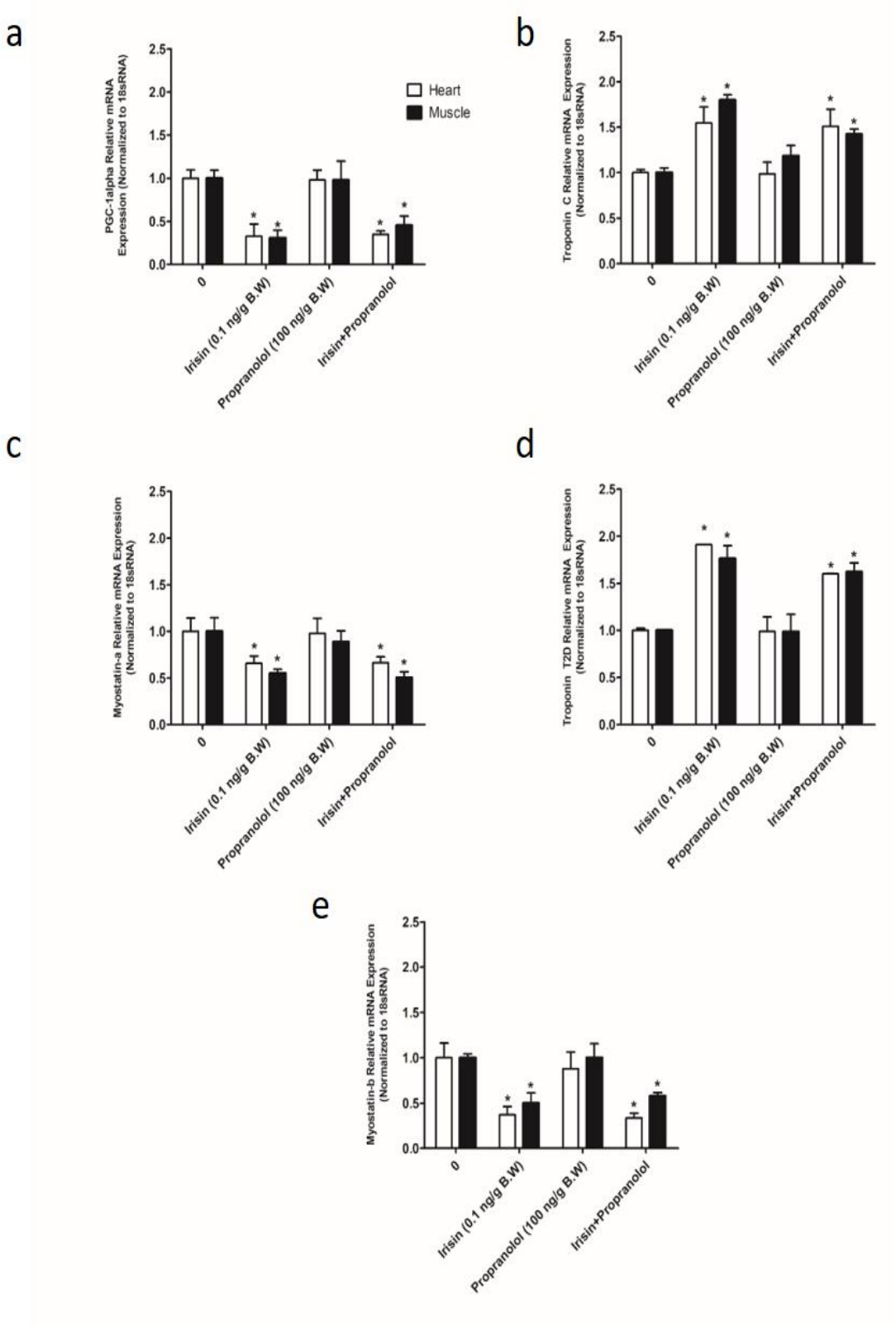

a
PGC-1alpha Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
0
Irisin (0.1 ng/g B.W)
Propranolol (100 ng/g B.W)
Irisin+Propranolol
b
Troponin C Relative mRNA Expression (Normalized to 18sRNA)
c
Myostatin-a Relative mRNA Expression (Normalized to 18sRNA)
d
Troponin T2D Relative mRNA Expression (Normalized to 18sRNA)
e
Myostatin-b Relative mRNA Expression (Normalized to 18sRNA)

Figure 6.3. Irisin, irisin and propranolol had similar effects on regulation of muscle proteins in zebrafish. Groups include: Control, Irisin (0.1 ng/g B.W), Propranolol (100 ng/g B.W), Irisin + Propranolol. Heart (white bars) and muscle (black bars) and the relative mRNA expression of muscle proteins were normalized to 18s RNA. Asterisks denote significant differences between control group and other groups. Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison tests were used for statistical analysis (*$P<0.05$; Compared to control, N=6 fish/group).

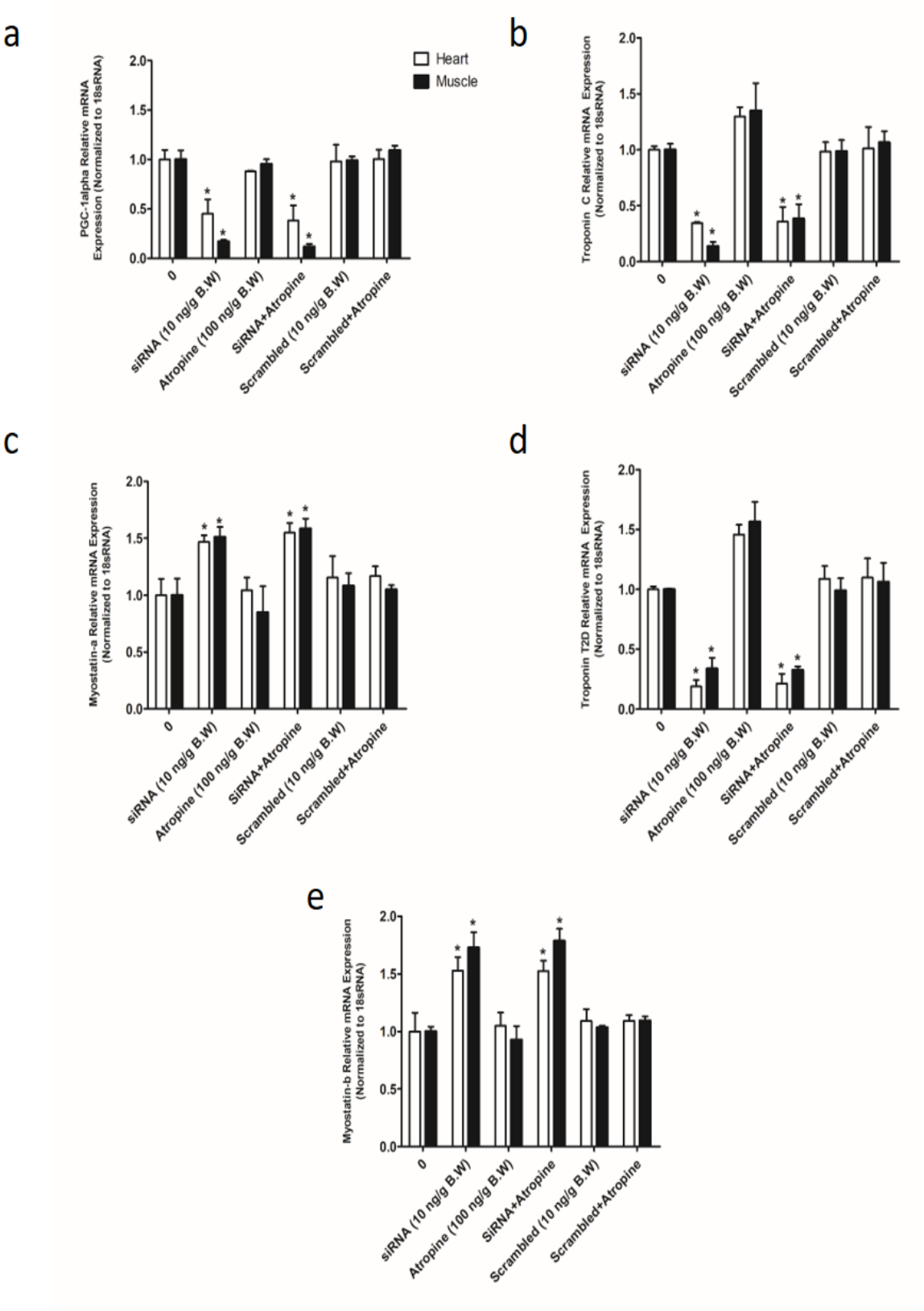

a
PGC-1alpha Relative mRNA Expression (Normalized to 18sRNA)
Heart
Muscle
2.0
1.5
1.0
0.5
0.0
0
siRNA (10 ng/g B.W)
Atropine (100 ng/g B.W)
SiRNA+Atropine
Scrambled (10 ng/g B.W)
Scrambled+Atropine
b
Troponin C Relative mRNA Expression (Normalized to 18sRNA)
c
Myostatin-a Relative mRNA Expression (Normalized to 18sRNA)
d
Troponin T2D Relative mRNA Expression (Normalized to 18sRNA)
e
Myostatin-b Relative mRNA Expression (Normalized to 18sRNA)

Figure 6.4. Administration of atropine either alone or in the presence of irisin siRNA did not affect the mRNAs encoding muscle proteins in zebrafish. Groups include: Control, Irisin siRNA (10 ng/g B.W), Atropine (100 ng/g B.W), Irisin siRNA + Atropine, Scrambled siRNA (10 ng/g B.W) and Scrambled siRNA + Atropine. Heart (white bars) and muscle (black bars) and the relative mRNA expression of muscle proteins were normalized to 18s RNA. Asterisks denote significant differences between control group and other groups. Data are represented as mean + SEM. One-way ANOVA followed by Tukey's multiple comparison tests were used for statistical analysis (*$P<0.05$ Compared to control; N=6 fish/group).

6.4. Discussion

Our previous studies showed that irisin regulates cardiac physiology and modulated mRNAs of the muscle proteins in zebrafish (**Chapter 5**). *In vivo* administration (i.p) of irisin elicited an overall stimulatory effect on cardiovascular function in zebrafish. ICV injection of irisin into the third ventricle of brain activated the hypothalamic neurons and increased blood pressure and cardiac contractility in rats [22]. Peripheral injections of irisin protected the heart against ischemia and perfusion injury in C57/BL6 mice [316]. Microinjection of irisin into the nucleus ambiguus caused a decrease in blood pressure and elicited a cardioprotective role in Sprague-Dawley rats. *In vitro* studies showed that irisin activated the nucleus ambiguus neurons of Sprague Dawley rats resulting in increased intracellular Ca2+ concentration levels, an important regulator in cardiac contraction [274, 378]. Collectively, irisin is a regulator of cardiac physiology in zebrafish.

This study focused on determining the potential mechanisms of irisin effects on cardiovascular biology. We explored whether autonomic nervous system could possibly mediate the cardiovascular effects of irisin. The adrenergic nervous system (or sympathetic nervous system) elicits a wide variety of cardiovascular effects that includes increase in heart rate and cardiac contractility, whereby it improves cardiac performance and preparations for the fight or flight response [171]. In contrast, the parasympathetic nervous system (cholinergic system) plays an important role in slowing the heart rate (bradycardia) and decreasing cardiac muscle contractility. For understanding the mechanism underlying the effects of irisin in mediating cardiac functions in zebrafish, propranolol, a non-cardioselective β-adrenoceptor antagonist (blockade of sympathetic pathway) and atropine sulfate, a muscarinic acetylcholine receptor

antagonist was considered [363, 370, 373, 379]. Administration of propranolol (100 ng/g B.W) decreased heart rate, cardiac output, end diastolic and systolic volumes in zebrafish, while co-injection of propranolol with irisin (0.1 ng/g B.W.) reversed these irisin effects (**Figure 6.1 A-E**). The effects of propranolol alone found in this study are comparable to results reported previously. Exposure of propranolol (0.1 µg/L) reduced heart rate in zebrafish and Japanese medaka embryos [187], and affected cardiac performance and cardiac morphology in zebrafish embryos. In addition to these, cocaine-induced tachycardia (increased heart rate) was blocked by propranolol in zebrafish [380]. In water-breathing Asian swamp eels, administration of propranolol reduced heart rate, which led to bradycardia [381]. In white sturgeon, administration of propranolol significantly decreased cardiac output, gut blood flow, mean arterial pressure and heart rate during hypercarnia conditions [382]. Propranolol lowered heart rate and blocked the positive chronotropy induced by isoproterenol at all stages of development in rainbow trout [383]. Similar to these findings, the cardio stimulatory effects of irisin were blocked by propranolol, indicating that irisin regulates cardiovascular function via sympathetic stimulation in zebrafish. Overall, the results from this study provide evidence for one critical mediator of irisin effects on cardiac function in zebrafish. What remains unclear is whether irisin directly stimulates beta1-adrenergic receptors or instead (more likely) has an effect to increase sympathetic nervous system activity.

I.P injection of atropine (100 ng/g B.W) alone increased heart rate and cardiac output in zebrafish (**Figure 6.2 D and E**). Meanwhile, atropine was not effective in modulating the cardiac effects of irisin knockdown using siRNA. In European sea bass, administration of atropine alone increased heart rate and cardiac output but decreased stroke volume [384]. During the

postprandial period in European sea bass, atropine administration increased heart rate without affecting oxygen consumption, while infusion of propranolol normalized the cardiac function and oxygen consumption in European sea bass [384]. In water-breathing eels, atropine administration caused an increase in heart rate leading to tachycardia [381]. In addition to that, administration of atropine increased mean heart rate, while infusion of propranolol and/or atropine caused a decline in heart rate and cardiac output in African sharptooth catfish [385]. Cardio-stimulatory effects of atropine sulfate are at least in part, mediated by inhibition of ACh by opposing the actions of the vagus nerve in smooth muscle leading to increased effects on heart rate and contractility causing tachyarrhythmias [371, 386, 387]. Atropine is known to work as an anti-parasympathetic agent by competitive binding to muscarinic receptors with ACh. [27, 371]. However, the cardiac effect by atropine was ineffective in this thesis to compensate for the loss/decrease (siRNA effect) of endogenous irisin. Parasympathetic system likely plays no role in mediating the cardiovascular changes when the irisin milieu is compromised in zebrafish. Thus, the physiological mechanism by which irisin knockdown suppresses cardiac function remains unanswered.

In an attempt to understand the possible mechanisms of autonomic nervous system blockers on muscle proteins, we then measured the gene expression of muscle proteins that were found to change in response to irisin from our previous studies (Chapter 5). Our *in vitro studies* revealed that irisin in the presence of propranolol downregulated PGC-1 alpha, myostatin-a and b relative mRNA expression in zebrafish. The reduction in the expression of PGC-1 alpha was in accordance with our previous studies in fish [306]. Irisin in the presence of propranolol upregulated troponin C and T2D mRNA relative mRNA expression in zebrafish heart and

skeletal muscle which was in accordance with our previous studies that irisin administration upregulated troponin C and T2D relative mRNA expression in zebrafish [306]. Troponin C and T2D, two cardiac proteins have shown to play an important role in maintaining the actin-myosin complex and regulating the release of Ca2+ ions from the sarcomere in muscle contraction [218, 354]. In addition to that, irisin siRNA in the atropine did not have a significant effect in regulating muscle proteins when compared to effect of muscle proteins upon irisin siRNA administration [306].

In conclusion, this research identified a role for the adrenergic pathway in regulating irisin effects on cardiovascular physiology in zebrafish. However, blockade of sympathetic pathway (propranolol) did not influence muscle proteins that were modulated by irisin in zebrafish (**Chapter 5**). The cardiovascular effects were measured at 15 min post-injection, while the mRNAs were measured at 1 hour post-injection. It is possible the effects of irisin on these mRNAs is a direct effect on cardiomyocytes, while the physiological effects on heart function is primarily mediated by the sympathetic system. Our results also show no interaction of irisin and the parasympathetic system, at least at the doses tested. Additional studies, including the measurement of catecholamines during exogenous irisin or irisin knockdown, are required to gain more mechanistic insights on irisin effects and the autonomic nervous system in fish.

Chapter 7

General Discussion

The main aims of my thesis research were to characterize NLP and irisin in fish, and to determine whether these are novel regulators of feeding and cardiovascular functions in zebrafish and goldfish. Overall, these goals were successfully completed, and significant set of data supporting my hypothesis is provided in chapters 2-6. The main contributions of this research, key limitations, and some future directions are outlined in this chapter.

7.1. Contribution I: Identification of NLP as a Novel Anorexigen in Goldfish

The research outlined in Chapter 2 presents several key discoveries on NUCB1/NLP biology in goldfish (**Figure 7.1**). Our *in silico* analysis found that NUCB1 in fish and mammals encode a nesfatin-1-like sequence and possesses prohormone convertase sites that should facilitate its processing [220, 227]. RT-qPCR, Western Blot analysis and immunohistochemical studies showed NUCB1 expression in both central and peripheral tissues in goldfish [15], providing supportive evidence for a possible metabolic effect for NLP. Pituitary NUCB1 mRNA expression was downregulated by estradiol and upregulated by testosterone in female goldfish brain. NUCB1 mRNA was modulated by macronutrients and displayed a daily rhythmic pattern of expression in goldfish [15]. The negative modulation of NUCB1 upon food deprivation suggests an anorexigenic action for NUCB1 and/or encoded NLP. Our next study determined if NLP is indeed biologically active in goldfish. I.P administration of NLP (10 ng/g and 100 ng/g B.W) caused potent inhibition of food intake. NLP injection downregulated mRNAs encoding

orexigens, orexin-A and ghrelin and upregulated CART mRNA expression in goldfish brain [15]. Collectively, these results provide first set of evidence supporting a naturally occurring NLP, hormonal and external regulators of its expression, and an anorectic action for NLP. The addition of NLP to the growing list of metabolic factors increases the complexity associated with the regulation of energy balance. **Figure 7.3** shows how NLP fits into the general model of complex and redundant milieu that regulated food intake in teleost fish. The localization of NUCB1 and NLP in tissues involved in metabolic regulation, and its action on at least some of the orexigens and anorexigens provide supportive evidence for a prominent role for NLP in feeding regulation in fish.

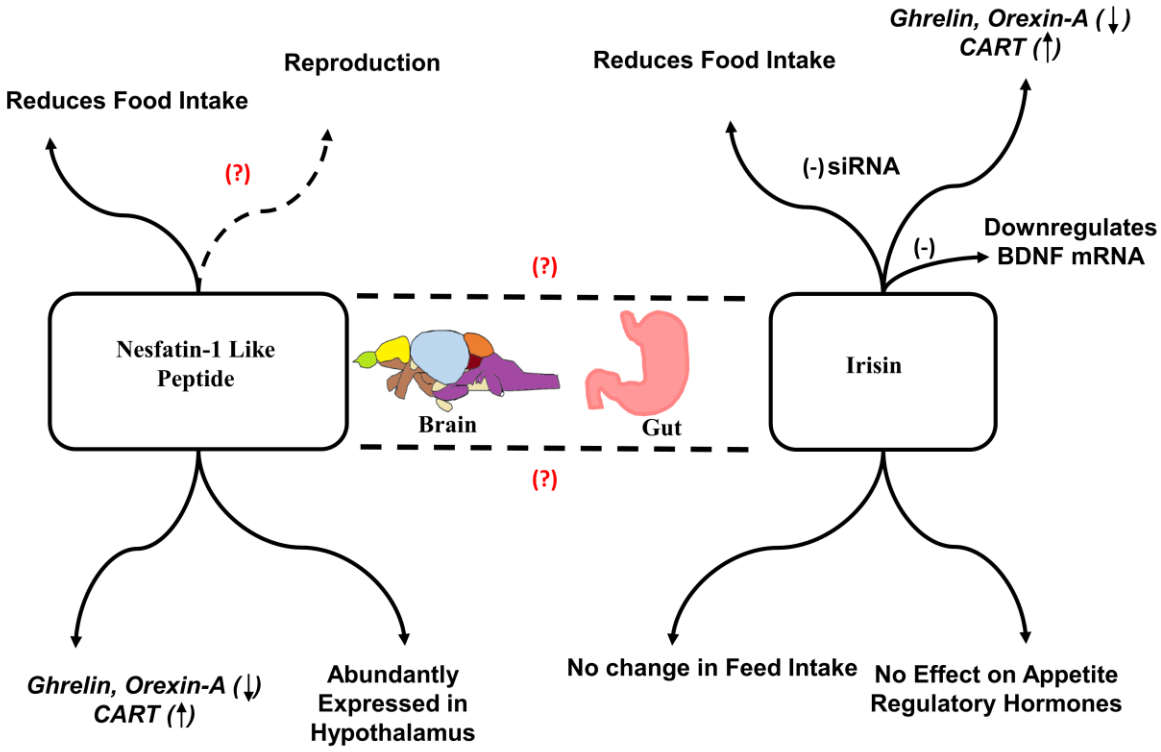

Reproduction
Reduces Food Intake
(?)
Nesfatin-1 Like Peptide
Ghrelin, Orexin-A (↓)
CART (↑)
Abundantly Expressed in Hypothalamus
Brain
Gut
(?)
(?)
Reduces Food Intake
Ghrelin, Orexin-A (↓)
CART (↑)
(-) siRNA
(-)
Downregulates BDNF mRNA
Irisin
No change in Feed Intake
No Effect on Appetite Regulatory Hormones

Figure 7.1. NLP and irisin regulation of feeding in fish. Summary depicting the role of NLP and irisin in regulating feed intake in fish. NLP is abundantly expressed in the hypothalamus and has a negative role in regulating feed intake. In brain and gut, NLP downregulated orexigens (Ghrelin and orexin-A mRNA; indicated by downward arrow in brackets) and upregulated anorexigens (CART mRNA) (indicated by upward arrow). Whether NLP has a role in reproduction in fish remains unknown (indicated by dotted arrows). On the contrary, irisin, a skeletal muscle protein had no significant effect on feeding and appetite regulatory proteins in zebrafish. Knockdown of irisin by siRNA had a suppressive effect on feeding, downregulated orexigens (Ghrelin and orexin-A mRNA) (indicated by downward arrow) and upregulated anorexigens (CART mRNA) (indicated by upward arrow) in zebrafish. Knockdown of irisin also downregulated BDNF mRNA expression in brain, heart, muscle and gut of zebrafish. Whether NLP and Irisin interact with each other in brain and gut to regulate feeding in fish warrant further studies (indicated by dotted arrows). NLP - Nesfatin-1-Like Peptide; CART - Cocaine-and amphetamine-regulated transcript; BDNF - Brain-derived neurotrophic factor.

Limitations and Future Directions: While the predicted NLP sequence and PC cleavage sites are present in the NUCB1 sequences, it is not confirmed that NLP is indeed produced and secreted from fish tissues. Additional studies using immunoprecipitation and MALD-MS analysis are required to confirm this. For immunohistochemical studies and Western blot analysis the NUCB1 antibody used (Pacific Immunology) is raised in rabbit against mouse. In some of our studies, this antibody was not effective in detecting signals and cell-specific expression, although multiple bands and smearing in Western gels were detected. It is likely that we have to use an antibody specifically raised against fish species to further enrich our data in understanding cell-specific expression and protein localization using immunohistochemical studies and Western blot analysis. The current research design considered only one type of administration of the peptide (I.P. injection), and that again measured only an acute effect. In addition to this, central administration and long-term studies using continuous infusion should be tested to identify chronic effects of NLP on food intake, body weight and metabolic partitioning. The mechanism of action of NLP in fish tissues also warrants further consideration. In this study, and in the following chapters, mixed sex (both males and females) was randomly used for tissue distribution and physiological studies. It is possible that there is sexual dimorphism in the expression and function of peptides studied here. Studies should be conducted in male and female fishes separately to identify potential sex specific differences in peptide function.

7.2. Contribution II: NLP is a Cardio-Suppressor That Affects Calcium Handling Proteins Encoding mRNAs in Zebrafish

Since NLP and nesfatin-1 sequences share high similarities, especially in the putative bioactive core region, it is possible that they both utilize the same receptors and share common effects in the regulation of homeostasis. My studies described in Chapter 3 provide novel evidences on NLP action on cardiovascular physiology in zebrafish **(Figure 7.2).** I.P. administration of zebrafish and rat NLP (10 ng/g and 100 ng/g B.W) reduced cardiac functions in zebrafish and goldfish. These results are in agreement with previous findings of nesfatin-1 effects on cardiac physiology in zebrafish. Both nesfatin-1 and NLP elicit similar effects in regulating cardiovascular functions in zebrafish and goldfish. NLP injection downregulated irisin and RyR1b mRNA expression in zebrafish. However, ATP2a2 mRNA expression was elevated in the heart of NLP treated fish (**Figure 7.2**). Irisin, a skeletal muscle peptide, plays an important role in stimulating cardiac function in mice [316]. More recently, irisin has gained importance as a potential biomarker for myocardial infarction due to its abundance in cardiac and skeletal muscle [39, 237]. Irisin downregulated NUCB1 mRNA expression in zebrafish heart and muscle (**Figure 7.2**). Our previous results reported that irisin has a positive role in regulating cardiac function and modulates muscle proteins in zebrafish [306]. In conclusion, NLP is a cardio-suppressor in zebrafish and modulates calcium handling proteins in zebrafish (**Figure 7.2**). Cortisol is critical for activation of autonomous nervous system (ANS), which in turn regulates cardiovascular physiology [388, 389]. We found that NLP elevated cortisol levels and modulated stress related hormones in goldfish (**Appendix A**). It is possible that the stress axis and its hormones are possible mediators of NLP effects on cardiac functions in fish. In addition, NLP administration downregulated irisin relative mRNA expression in zebrafish heart and skeletal

muscle. It is possible that NLP modulates cardiac activity by inhibiting the action of irisin in zebrafish. Further studies need to be elucidated to understand the mediators and cellular mechanisms of cardiac action of irisin in zebrafish.

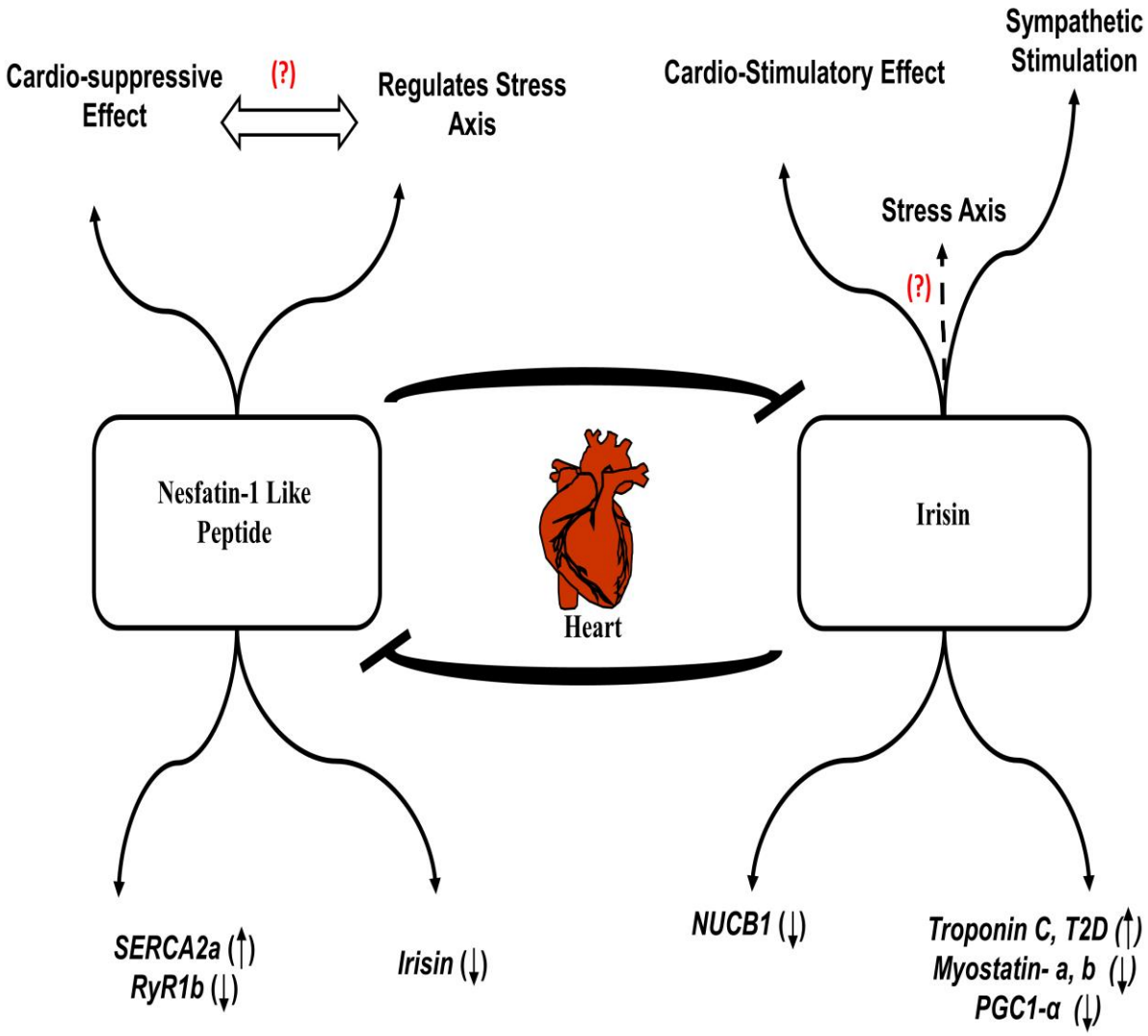

Cardio-suppressive Effect
(?)
Regulates Stress Axis
Cardio-Stimulatory Effect
Sympathetic Stimulation
Stress Axis
(?)
Nesfatin-1 Like Peptide
Heart
Irisin
SERCA2a (↑)
RyR1b (↓)
Irisin (↓)
NUCB1 (↓)
Troponin C, T2D (↑)
Myostatin- a, b (↓)
PGC1-α (↓)

Figure 7.2. Summary of NLP and irisin effects on cardiac function and regulation of muscle proteins in fish. NLP, an anorexigenic peptide has a cardio-suppressive effect in zebrafish and goldfish. In addition to that, NLP affected calcium binding proteins in zebrafish. NLP downregulated irisin mRNA (indicated by down arrow) in fish. In addition to that, NUCB1/NLP is modulated by stress, and NLP has effects on the HPI axis in regulating cortisol secretion in goldfish. On the other hand, irisin, a myokine has a positive role in regulating cardiac functions in zebrafish. Adrenergic system mediates irisin regulation of cardiovascular effects in zebrafish. Irisin plays an important role in modulating muscle proteins encoding mRNAs and downregulated NUCB1 mRNA expression in zebrafish. Whether irisin modulates stress axis (indicated by question mark) and that stress axis is important for regulating cardiovascular function warrant further studies (indicated by question mark). NLP- Nesfatin-1-Like Peptide; T2D- Troponin T2D; PGC-1α- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NUCB1- Nucleobindin-1; SERCA2a- Sarco/endoplasmic reticulum Ca^{2+}-ATPase; RyR1b- Ryanodine receptor 1b.

Limitations and Future Directions: My studies only determined acute effects on cardiac function, and only mRNA expression was measured. Future studies should consider longer term effects of NLP on cardiac functions. In addition, Western blot analysis and imaging tools should be used to determine the expression and function of calcium handling proteins and calcium homeostasis in cardiomyocytes. The current study showed that NLP is a cardio-suppressor in goldfish and zebrafish (**Figure 7.4**). However, the mechanisms and pathways behind this action remain poorly understood. We also determined that adrenergic system mediates irisin action on cardiac function in zebrafish. Does NLP also regulate cardiac function by autonomic nervous system in zebrafish warrant further studies. In future, beta-adrenergic blockers (propranolol and atropine) can be administered in combination with NLP (effective dosage) to study whether NLP mediates cardiac functions via autonomous nervous system in fish and a possible relationship between NLP and irisin in fish. The role of NLP in cardiac functions after meal intake is also another area that requires attention.

7.3. Contribution III: Endogenous Irisin is Essential for Maintaining Food Intake in Zebrafish

The research outlined in Chapter 4 describes the role of endogenous irisin in regulating food intake in zebrafish **(Figure 7.1)**. Irisin is a myokine encoded in FNDC5. FNDC5 forms an integral part of the muscle post-exercise and causes an increase in energy expenditure in mammals. If irisin is released post-exercise, does it cause any anabolic or catabolic effects? The goal of this research was to determine whether irisin influences feeding and regulates appetite regulatory peptides in zebrafish [361]. Intraperitoneal injection of irisin did not affect feeding, but its knockdown using siRNA caused a significant reduction in food intake [361]. Knockdown of irisin reduced ghrelin and orexin-A mRNA expression, and increased CART mRNA expression in zebrafish brain and gut [361]. These peptides that were found altered by the absence of endogenous irisin are possible mediators of its effects on feeding (**Figure 7.1**). Brain derived neurotrophic factor (BDNF) expression, a neurotrophin and anorexigen in rodents, was also determined after siRNA based knockdown of irisin [335, 390]. siRNA mediated knockdown of irisin downregulated BDNF mRNA in zebrafish. The role of BDNF on food intake in fish is unknown. The above results agree with previous findings that showed irisin induced BDNF mRNA expression in humans and goldfish [330]. These results indicate that unaltered endogenous irisin is required to maintain food intake in zebrafish (**Figure 7.1; 7.3**).

Limitations and Future Directions: Our research showed that knockdown of irisin by siRNA reduced feed intake in zebrafish. To provide additional confirmation for this finding, irisin should be reintroduced by I.P injection. If the regulation of feed intake caused by irisin knockdown are masked after the administration of irisin, it will provide further evidence to

confirm that it was indeed the absence of endogenous irisin that caused a decrease in food intake in zebrafish. Whether macronutrients alter endogenous irisin levels in fish remain unclear. Our future studies will focus on feeding the fish with special diets containing varying amounts of macronutrients to understand the relationship between irisin and feeding in fish. In order to supplement the current results that irisin regulates feed intake in fish, food deprivation and periprandial studies can be carried to understand the relationship of irisin and nutrient status in fish. Our future studies should focus on measuring irisin in fish subjected to food deprivation (1,2,7,14 days) versus fish that are *ad libitum* fed. In addition, periprandial (-3 to +3 hours) pattern of endogenous irisin has to be quantified to determine the profile of irisin in response to a meal. We did not measure endogenous irisin (processed peptide) and only one mode of administration was used for testing its effects on feeding. In the irisin (chapters 4-6) and NLP (chapters 2-3) characterization achieved in my research, we did not measure circulating concentrations of these peptides. It is important to understand the levels of endogenous irisin and NLP to determine how physiologically relevant the doses we tested are. Some of the doses chosen for injections are possibly above the naturally occurring levels of endogenous irisin and NLP. Thus, the effects observed after exogenous irisin and/or NLP, at least in part are pharmacological, not physiological.

7.4. Contribution IV: Irisin Regulates of Cardiac Physiology and Cardiac Proteins in Zebrafish

Having determined the role of irisin on feeding, and its action on metabolic peptides (Chapter 4), we then tested whether irisin regulates cardiac physiology in zebrafish (Chapter 5). Western blot analyses detected the presence of irisin protein (23 kDa) in zebrafish heart and skeletal muscle, and irisin immunoreactivity was detected in both tissues. *In vitro* studies found that treatment with irisin (0.1 nM) downregulated the expression of PGC-1 alpha, myostatin a, and b, while upregulating troponin C mRNA expression in zebrafish heart and skeletal muscle [306]. Exogenous irisin (0.1 and 1 ng/g B.W) increased diastolic volume, heart rate and cardiac output, while knockdown of irisin (10 ng/g B.W) showed opposing effects on cardiovascular function (**Figure 7.2**). Administration of irisin (ICV) increased cardiac output and blood pressure in rats by activating hypothalamic neurons, while peripheral (intravenous) administration into the nucleus ambiguus decreased blood pressure, decreased cardiac function and caused vasodilation [22, 274]. Irisin (1 and 10 ng/g B.W) downregulated PGC-1 alpha, myostatin a and b, and upregulated troponin C and troponin T2D mRNA expression (**Figure 7.2**). Meanwhile, knockdown of irisin showed opposite effects on troponin C, troponin T2D and myostatin a and b mRNAs in zebrafish heart and skeletal muscle [306]. Collectively, these results identified muscle proteins as novel targets of irisin and added irisin to the list of peptide modulators of cardiovascular physiology in zebrafish (**Figure 7.4**).

Limitations and Future Directions: We only measured the expression of mRNAs encoding cardiac proteins of interest. Protein abundance must be studied to have more clarity in irisin effects on cardiac regulatory peptides. Western blot analysis showed the absence of FNDC5 in

siRNA knockdown samples. Pharmacological reintroduction of irisin in siRNA treated fish should be considered to confirm the results of our current findings on cardiac functions of irisin in zebrafish. Only a single time point (15 mins post injection) was used for cardiac measurements in this sturdy. An effective dosage should be considered and tested for 30, 45 and 60 min to time dependent cardiac effects of irisin in zebrafish. Tissue specific or whole-body knockout strategies should be used to delete endogenous irisin and determine how the cardiovascular system performs in its absence is also required.

7.5. Contribution V: Adrenergic System Mediates Irisin Effects on Cardiovascular Physiology in Zebrafish

Based on our results explained in chapter 5, we wanted to determine potential mechanisms of irisin effects on cardiovascular biology in zebrafish. To address this, whether adrenergic pathways mediate the cardiovascular effects of irisin was studied. Propranolol either alone or in the presence of irisin (blockade of sympathetic pathway) decreased cardiovascular parameters in zebrafish. Blockade of sympathetic pathway (propranolol) did not influence muscle proteins that were modulated by irisin in zebrafish (**Chapter 5**). Irisin siRNA co-injected with atropine (beta-agonist) also downregulated cardiac function in zebrafish. In conclusion, it is clear that sympathetic pathway has a role in mediating irisin effects on cardiac functions in zebrafish (**Figure 7.2**).

Limitations and Future Directions: While a role for the adrenergic system was clear for mediating the cardiac effects of irisin, no effects were found in the expression of mRNAs encoding cardiac proteins. Further studies are required to gain a deeper understanding of irisin effects and its interplay with the sympathetic system. While the current studies tested in fish suggests that NUCB1/NLP and irisin are present in central and peripheral tissues, evaluating its levels in circulation under different physiological conditions is required. It is important to measure irisin after exercise, and post-feeding to determine whether there is any relationship between physiological status and endogenous irisin. Such studies will also help us determine whether irisin is indeed an exercise hormone, as reported in humans.

7.6. Conclusions

The aim of this research was to gain better understanding of two novel regulators, NLP and irisin in fish. All of the objectives set out at the beginning of my thesis research were met, and the results obtained, in general, supported my hypotheses. Our discoveries indicate that irisin and NLP are key functional contributors to maintain feeding and cardiac functions (**Figure 7.1; Figure 7.2**). Food intake and cardiac physiology are modulated by multiple hormones and naturally occurring factors (**Figure 7.3; Figure 7.4**). This research placed NLP and irisin in the already multifactorial milieu as a modulator of body function, and regulator of some of the already characterized hormones that maintain cardiac and metabolic homeostasis (**Figure 7.3; Figure 7.4**). NLP and irisin share many common functions and tissue specific expression with other hormones of importance. While some of our results are in agreement with similar studies in rodents or in fish, it appears that species-specific differences exist in NLP and irisin expression and actions in regulating feeding and cardiac functions. Both peptides studied here are orphan ligands awaiting identification of receptors. Our results provide the basis for the future physiological research, and that aimed to determine molecular and cellular mechanisms of action of irisin and NLP in fish. Overall, this thesis provides a large set of novel data that helped in better our understanding on NLP and irisin in fish. The outcome of the current research study are initial steps to make groundbreaking advances in the field of aquaculture. Further research is required to determine the role of NLP and irisin towards enhancing muscle mass, feed conversion efficiency, markers for cardiovascular diseases and stress, and its role in reproduction. The results of my research, collectively, provide foundational knowledge that could be explored for advances in the field of aquaculture. The implications of these novel peptides, irisin and NLP on aquaculture are diverse. For example, future research aimed to

generate information useful for aquaculture could determine the role of NLP and irisin in enhancing muscle mass, feed conversion efficiency, its use as markers for cardiovascular diseases and stress, and its role in reproduction. Such research will help aquaculture/fisheries to eventually develop and utilize irisin and NLP based strategies for enhancing yield from cultured fish.

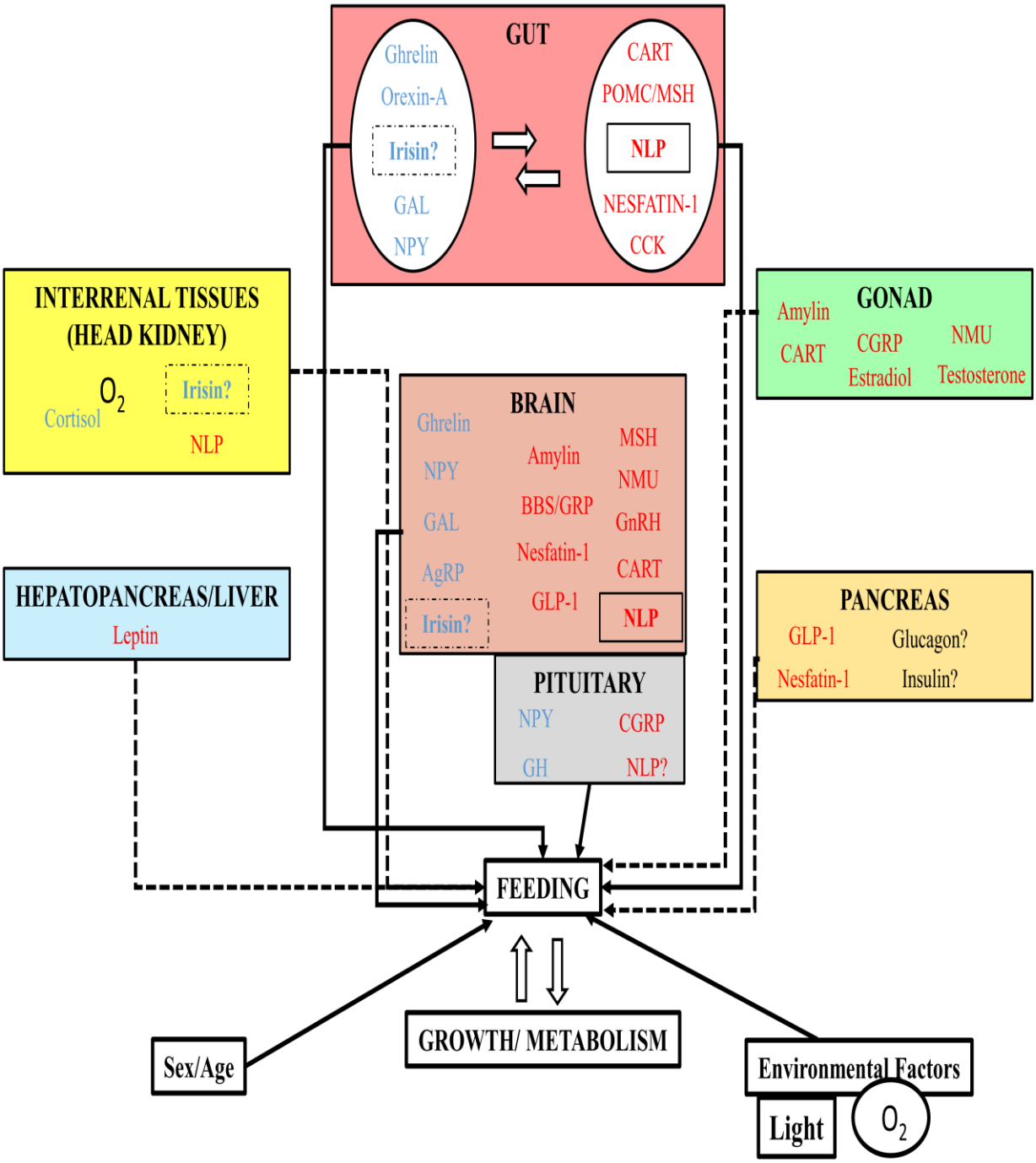

GUT
Ghrelin
Orexin-A
Irisin?
GAL
NPY
CART
POMC/MSH
NLP
NESFATIN-1
CCK
INTERRENAL TISSUES
(HEAD KIDNEY)
O2
Cortisol
Irisin?
NLP
GONAD
Amylin
CART
CGRP
Estradiol
NMU
Testosterone
BRAIN
Ghrelin
NPY
GAL
AgRP
Irisin?
Amylin
BBS/GRP
Nesfatin-1
GLP-1
MSH
NMU
GnRH
CART
NLP
HEPATOPANCREAS/LIVER
Leptin
PANCREAS
GLP-1
Nesfatin-1
Glucagon?
Insulin?
PITUITARY
NPY
GH
CGRP
NLP?
FEEDING
GROWTH/ METABOLISM
Sex/Age
Environmental Factors
Light
O2

Figure 7.3. Neuroendocrine factors regulating food intake in fish - Summary. Within the brain, central neuropeptide systems receive signals to regulate feed intake in fish. The factors that regulate feeding intake consist of feeding stimulators (orexigenic factors, Blue) and feeding inhibitors (anorexigenic factors, Red). Peripheral tissues comprising of liver, interrenal tissues, gut, and gonads send signals to brain and regulate the central neuropeptide signals to regulate feeding. The signals from periphery also travel via sensory axons in the vagus nerve and/or reach the brain via blood-brain barrier. Irisin and NLP are novel additions to the already available scheme, and both peptides regulate at least some other factors. Arrow heads represent a link between feeding and the central and peripheral tissues. GAL-Galanin; NPY- Neuropeptide Y; CART- Cocaine-and amphetamine-regulated transcript; CCK- Cholecystokinin; NLP- Nesfatin-1-Like Peptide; AgRP- Agouti-related protein; BBS/GRP- Bombesin/gastrin-releasing peptide; MSH- Melanocyte-stimulating hormone; GnRH- Gonadotropin-releasing hormone; GLP-1- Glucagon-like peptide-1; GH- Growth hormone; NMU- Neuromedin U; CGRP- Calcitonin gene-related peptide; O_2- Oxygen.

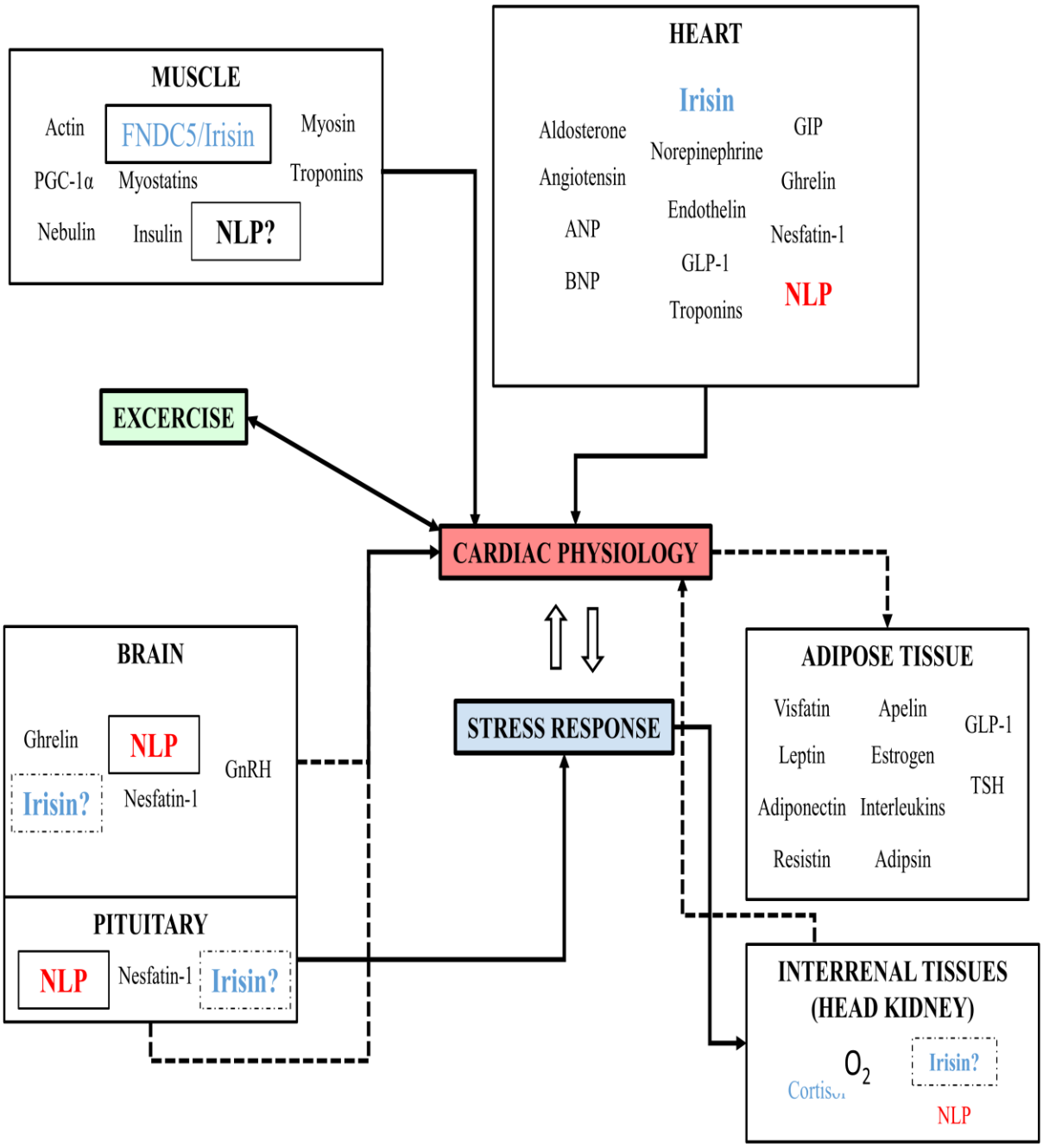

HEART
Irisin
Aldosterone
Norepinephrine
GIP
Angiotensin
Ghrelin
Endothelin
ANP
Nesfatin-1
GLP-1
BNP
NLP
Troponins
MUSCLE
Actin
FNDC5/Irisin
Myosin
PGC-1α
Myostatins
Troponins
Nebulin
Insulin
NLP?
EXCERCISE
CARDIAC PHYSIOLOGY
STRESS RESPONSE
BRAIN
Ghrelin
NLP
GnRH
Irisin?
Nesfatin-1
PITUITARY
NLP
Nesfatin-1
Irisin?
ADIPOSE TISSUE
Visfatin
Apelin
GLP-1
Leptin
Estrogen
TSH
Adiponectin
Interleukins
Resistin
Adipsin
INTERRENAL TISSUES
(HEAD KIDNEY)
O2
Irisin?
NLP

Figure 7.4. Hormonal regulators of cardiovascular physiology in fish. Scheme showing central and peripheral peptides known to regulate cardiovascular function in fish. These peptides could elicit direct effects on cardiac tissues to regulate it function. One of the main factors that regulate cardiac function is stress. Peripheral hormonal signals from heart, muscle, interrenal tissues and adipocytes reach the brain and influence the central neuropeptide signals during the period of stress to help the body's fight or flight response. Depending on the stress response, multiple factors have shown to regulate cardiovascular function in fish via the autonomous nervous system (sympathetic and parasympathetic stimulation). Autonomic nervous system in fish controls heart performance, gastric motility and also release of catecholamines via central and peripheral signals. In addition to that, upon endurance training/ exercise can stimulate the release of adrenaline which stimulates adrenergic neurons in teleost fish. The adrenergic transmitters act as adrenergic receptors (α or β type) in the effector organs to regulate cardiac performance in fish. Arrowheads represents a relationship between the central and peripheral organs in regulating cardiac milieu in fish. PGC-1α- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NLP- Nesfatin-1-Like Peptide; FNDC5- Fibronectin type III domain-containing protein 5; ANP- Atrial natriuretic peptide; BNP- Brain natriuretic peptide; TSH- Thyroid-stimulating hormone; GnRH-Gonadotropin-releasing hormone; GLP-1- Glucagon-like peptide-1; GIP- Gastric inhibitory polypeptide; O_2- Oxygen.

APPENDIX A

NUCB1/NLP AND THE STRESS AXIS IN GOLDFISH

A.1. RATIONALE

The stress response in fish is regulated by hormones produced by the brain (hypothalamus), pituitary and interrenal tissues in fish [391, 392]. These tissues comprise the hypothalamo-pituitary- interrenal axis (HPI axis) in fish [391, 392]. Several studies have reported the role of stress hormones in regulating feed intake, reproduction and cardiovascular functions in fish [392, 393]. Due to the focus of this thesis research, we will briefly discuss the role of nesfatin-1-like peptide (NLP) in regulating stress axis in fish. Nesfatin-1 is an 82-amino acid anorexigenic protein encoded in its precursor nucleobindin-2 [220, 281]. Previous results from rats have shown that NUCB2/nesfatin-1 immunoreactivity is colocalized with corticotropin-releasing factor (CRF) in the hypothalamus, the feeding and regulatory control center of the brain [394, 395]. NUCB2/Nesfatin-1 like immunoreactivity was also observed in the pituitary of goldfish in the rostral pars distalis (RPD), the region where corticotropes are located [281]. I.C.V administration of nesfatin-1 increased plasma adrenocorticotropic hormone (ACTH) and corticosterone [395]. Administration of CRF antagonist, Astressin-B opposed the effects of nesfatin-1 on feeding in rats [396]. Collectively, nesfatin-1 is a multifunctional functional peptide in fish and regulates stress endocrine axis in fish [281]. More recently, NUCB1 gained attention due to its similarity with NUCB2 and nesfatin-1 [15, 227]. For example, our *in silico* analysis found that NUCB1 in fish and mammals encode a nesfatin-1 like sequence. Since NUCB1 has more similarity with NUCB2 and nesfatin-1 [15], we investigated whether NLP/NUCB1 regulate stress axis similar to nesfatin-1 in fish.

A.2. HYPOTHESIS

We hypothesize that NUCB1/NLP in fish is modulated by stress, and that NLP has effects on the HPI axis in regulating cortisol secretion in goldfish.

A.3. SPECIFIC OBJECTIVES

1: To study the cell-specific expression of NUCB1/NLP in the HPI tissues of goldfish with CRF-receptor 1 (CRF-R1) and ACTH

2: To determine the effects of stress on endogenous NUCB1/NLP in goldfish

3: To quantify HPI axis hormones and receptors after the peripheral administration of NLP.

A.4. MATERIALS AND METHODS

A.4.1. To study the cell-specific expression of NUCB1/NLP in the HPI tissues of goldfish

The presence and localization of the protein in different tissue sections of goldfish were detected by immunohistochemical (IHC) studies. For IHC studies, brain, hypothalamus, pituitary and head kidney were collected and sectioned. Hypothalamic sections were considered for possible co-localization of mouse anti-human CRF-R1 (MAB3930; R&D Systems, Minneapolis, MN; 1:50) and rabbit polyclonal anti-mouse nucleobindin-1 (Catalog # 1312- PAC- 02, Pacific immunology, Ramona, CA, 1:3000) primary antibody. Pituitary and head kidney sections were stained for ACTH (AB49585; Abcam, Cambridge, MA; 1:200) and NUCB1 and anti-rabbit GPCR12 (ab 75349, Cambridge, MA, 1:1000) and NUCB1. The slides were then washed with PBS and then incubated with respective secondary antibody for one-hour at room temperature. The slides were mounted on Vectashield medium containing DAPI dye (Blue, Vector Laboratories). The slides were imaged using a Nikon inverted microscope (L100) (Nikon DS-Qi1 MC camera, ON, Canada) and analyzed using NiS Elements imaging software (Nikon, Canada). Slides incubated with secondary antibody alone or preabsorbed with NUCB1/NLP antibody served as negative controls.

A.4.2. To determine the effects of stress on endogenous NUCB1, CRF-R1 and ACTH mRNA in goldfish

In order to determine whether NUCB1/NLP is regulated upon stress, Goldfish (n=6 fish/ group) was subjected to 10 min of netting stress, followed by 5 min of crowding and restraint stress. After this, they were moved to a container prior to anesthesia for tissue collection. Total RNA extraction and cDNA synthesis was performed as described earlier (**2.2.3**). Quantification

of NUCB1, Corticotropin-releasing hormone receptor 1 (CRF-R1) and Adrenocorticotropic hormone (ACTH) mRNA was determined by RT-qPCR to determine the effects of stress on endogenous NUCB1/NLP in goldfish. The primer sequences of ACTH, CRF-R1, G protein-coupled receptor 12 (GPCR-12) and NUCB1 mRNA are shown in the Appendix table (**A.1**), and the mRNA expression was normalized to 18s RNA (housekeeping gene).

Appendix Table A.1. Forward and reverse primers, and the annealing temperature used in PCR and RT-qPCR analyses of the expression of mRNAs of interest during the study in zebrafish.

Gene	Primer sequence (5'-3')		Annealing temperature (°C)
	Forward	Reverse	
CRF-R1	ACCTCATTGGCACCTGCTGG	CTCAGGCACCGGATGCTCC	60
ACTH	TTACGGTGGCTTCATGACCT	CCTTGGAATGAGAAGACC	60
GPCR-12	CAGCCCAGCTGTTGACTGTA	GGGCTTGCTGCTAAAAAGTG	60

A.4.3. To determine the effect of peripheral administration of NLP on cortisol levels in goldfish.

Goldfish/Zebrafish NLP (VPIDRNPDPPQEEKAEENVDTGLYYDRYLREVIEVLETD PHFREKLQTANTEDIKNGRLSKELDLVGHHVRTRLDEL) synthesized by Pacific Immunology (Ramona, CA) was intraperitoneally injected at different doses (0 and 10 ng/g B.W) in goldfish (n=6 fish/ group). Blood samples were collected into 1.5 ml Eppendorf tubes from goldfish by cutting off the caudal vein post 1-hour and 2-hour of injection. Serum was separated by centrifugation at 7,000 rpm for 9 min and stored at -80 °C for cortisol analyses. Cortisol levels in goldfish serum was quantified using a cortisol ELISA kit (Catalog # 500360, Cayman Chemicals, USA). Plates were read using a Vmax microplate reader (VWR, Radnor, USA) between 405-420 nm wavelength and analyzed using SoftMax Pro 5 software to determine the levels of cortisol upon NLP administration.

A.4.4. Does NLP regulate stress hormones in goldfish?

To determine the role of NLP in regulating stress hormones, goldfish were anesthetized post 1-hour NLP injection and brain, hypothalamus, pituitary, head kidney tissues were collected for further analysis. Total RNA extraction, cDNA synthesis and quantification of PCR were performed as described earlier (**2.2.3**) to determine whether CRF-R1, ACTH and GPCR-12 mRNA (**Appendix A.1**) are modulated by NLP administration by RT-qPCR.

A.4.5. Statistical Analysis

Data were analyzed using t-test. PRISM version 5 (GraphPad Inc., USA) and IBM SPSS™ version 21 (IBM, USA) were used for statistical analysis. $P < 0.05$ was considered statistically significant. Data are represented as mean + SEM.

A.5. RESULTS

A.5.1. NUCB1/NLP-like Immunoreactivity Colocalizes CRF-R1 Like Immunoreactivity in the Hypothalamus of Goldfish

NUCB1/NLP-Like Immunoreactivity was colocalized with CRF-R1 in the nucleus lateralis tuberis posterioris (NLT*p*) of goldfish hypothalamus (**A-D**). DAPI (blue color) stained the nuclei of the cells (**A, D, E**). No staining was found in negative controls stained with secondary antibody or preabsorption control in the hypothalamus tissues of goldfish (**E**).

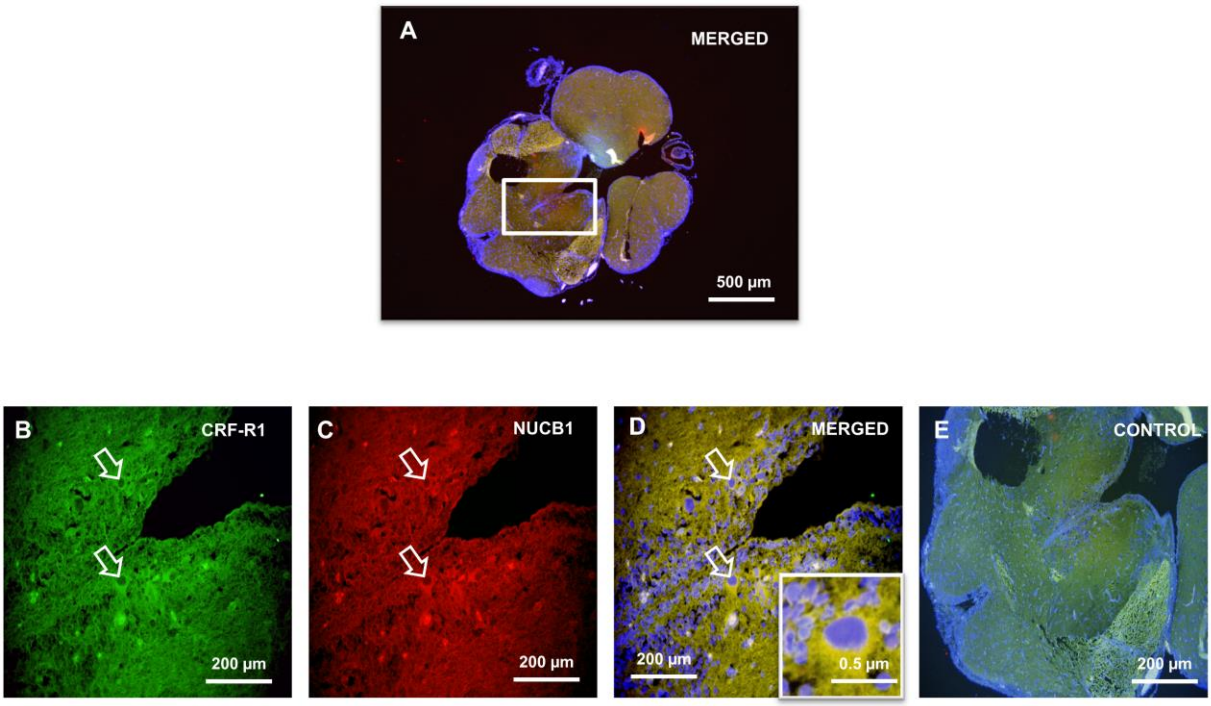


Figure A.1. Colocalization of NUCB1/NLP-like immunoreactivity and CRF-R1-like immunoreactivity in the goldfish hypothalamus. Colocalization of NUCB1/NLP-like Immunoreactivity and CRF-R1 was observed in hypothalamus of goldfish (**A, D**). Merged image shows colocalization of NUCB1 and CRF-R1 in goldfish hypothalamus (**D**). Preabsorption control of NUCB1/NLP was used as negative control **(E)**. Nuclei are stained blue (DAPI) (**A, D, E**), CRF-R1-like immunoreactivity (**B**; green), NUCB1/NLP (**C**; Red). Images were taken at 40X magnification and scale bar = 500 μm and 200 μm (and 0.5 μm for inset).

A.5.2. NUCB1/NLP-Like Immunoreactivity Colocalizes ACTH-Like Immunoreactivity in the Pituitary of Goldfish

NUCB1/NLP like immunopositive cells were colocalized with ACTH-like immunoreactivity (**A-D**). For negative control, the sections were stained with secondary body or preabsorption using synthetic goldfish NLP **(B, E**). DAPI (blue color) stained the nuclei of the cells (**A, D, E**).

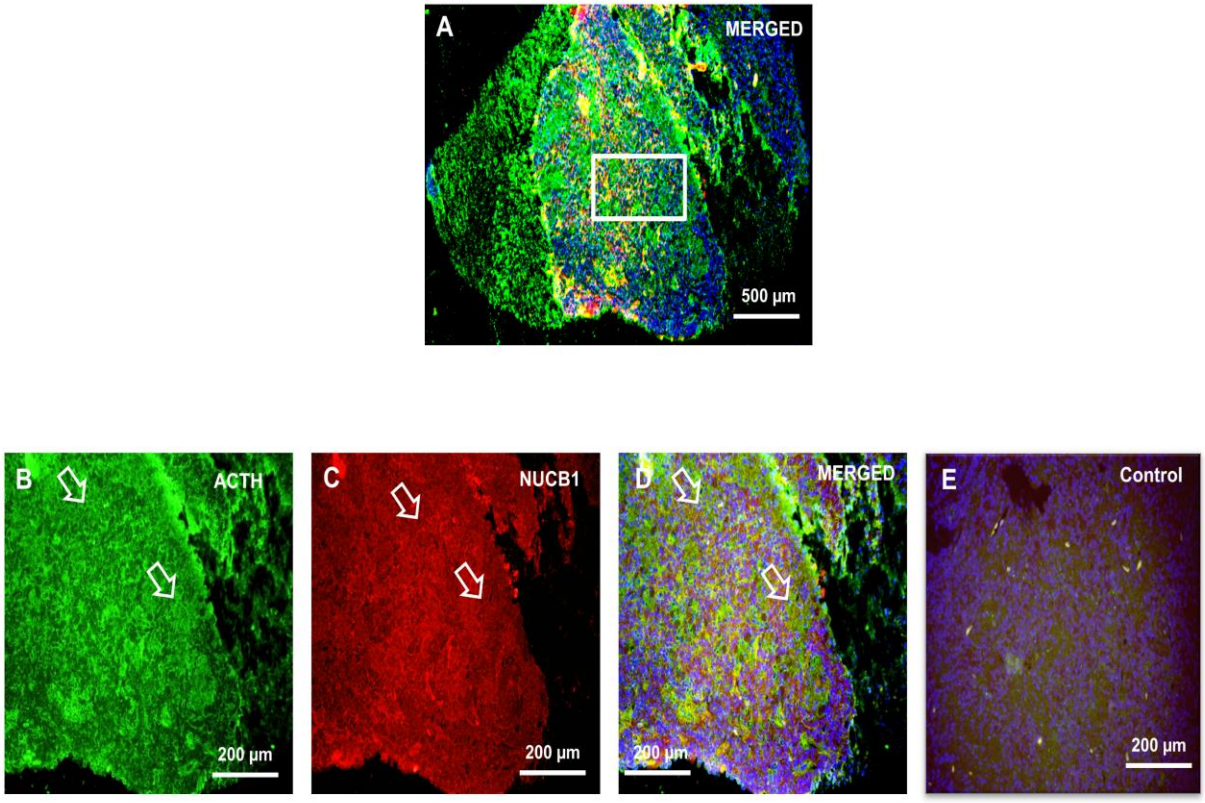


Figure A.2. Colocalization of NUCB1/NLP-like immunoreactivity and ACTH-like immunoreactivity in the goldfish pituitary. Colocalization of NUCB1/NLP-like Immunoreactivity and ACTH-like immunoreactivity was observed in pituitary of goldfish (**A, D**). Merged image shows colocalization of NUCB1 and ACTH in pituitary of goldfish (**D**). Preabsorption control of NUCB1/NLP was used as negative control **(E)**. Nuclei are stained blue (DAPI) (**A, D, E**), ACTH-like immunoreactivity (**B**; green), NUCB1/NLP (**C**; Red). Images were taken at 40X magnification and scale bar = 200 μm.

A.5.3. GPCR12-Like Immunoreactivity is colocalized with ACTH-Like Immunoreactivity in the Interrenal Cells of the Goldfish Head Kidney

GPCR12-like immunoreactivity was observed with ACTH-like immunoreactivity in distinct cytoplasmic vesicles of head kidney interrenal cells (**A-D**). Sections that were stained with the secondary body or preabsorption using synthetic goldfish NLP served as negative controls (**E**).

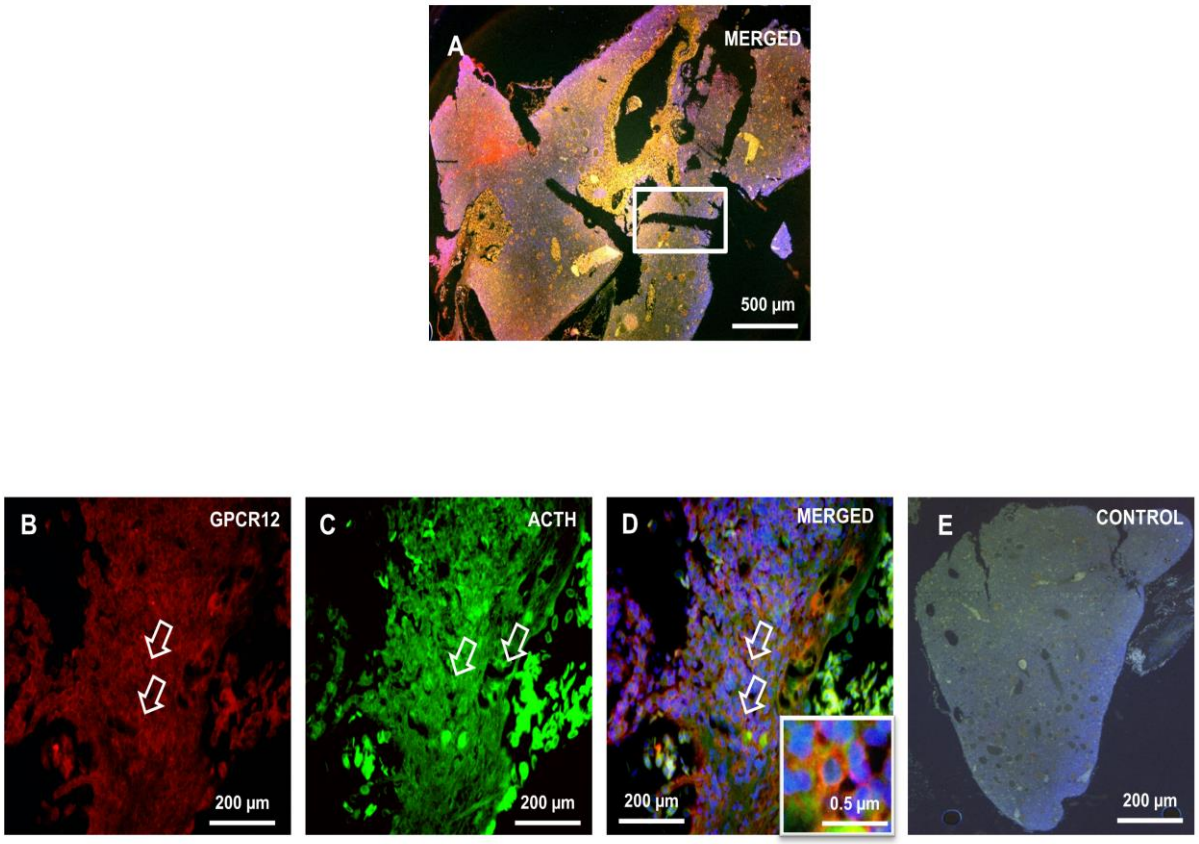


Figure A.3. Colocalization of GPCR12-Like Immunoreactivity and ACTH-like immunoreactivity in the interrenal cells of head kidney of goldfish. Colocalization of NUCB1/NLP-like Immunoreactivity and ACTH-like immunoreactivity was observed in pituitary of goldfish (**A, D**). Merged image shows colocalization of GPCR12 and ACTH in interrenal cells of head kidney (**D**). Preabsorption control of NUCB1/NLP was used as negative control **(E)**. Nuclei are stained blue (DAPI) (**A, D, E**), ACTH-like immunoreactivity (**C**; green), GPCR12 (**B**; Red). Images were taken at 40X magnification and scale bar = 500 μm and 200 μm (and 0.5 μm for inset).

A.5.4. Netting and Restraint Stress Increased Serum Cortisol Release in Goldfish

Goldfish that underwent netting and restraint stress for a period of 15 min showed significant increase in the serum cortisol levels when compared to control goldfish (P value – 0.031) (**Figure A.4**).

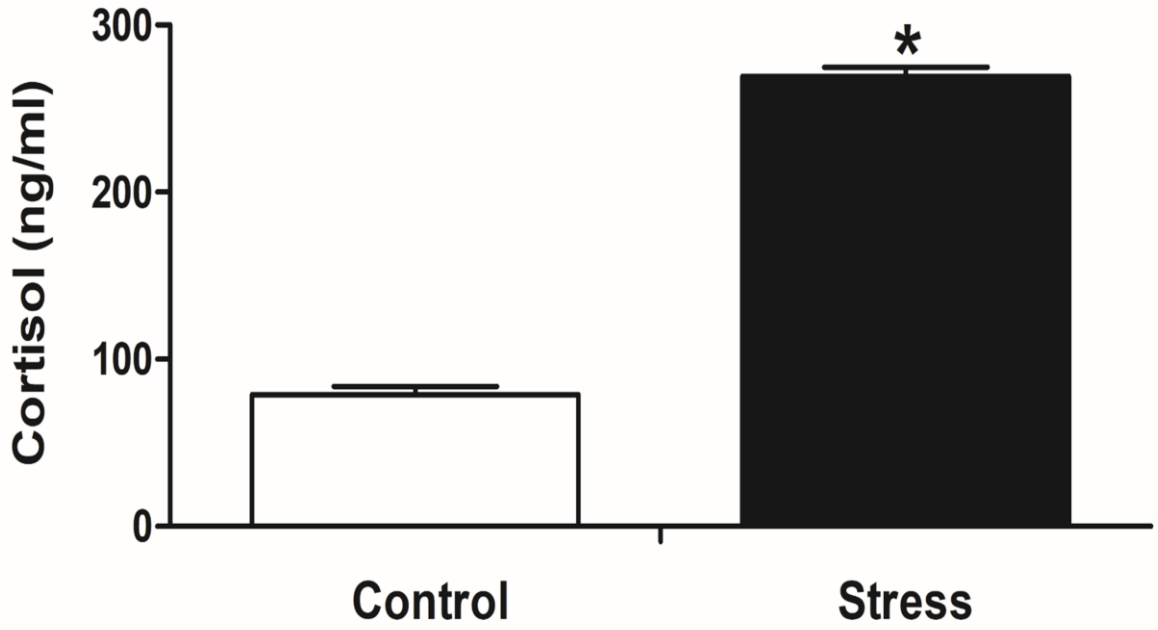


Figure A.4. Netting and restraint stress elevated the levels of serum cortisol levels in goldfish. Groups include: Control (white bar) and Stressed (Black bar) goldfish and the cortisol levels were measured in ng/ml. Asterisks denote significant differences between control (saline group) and stressed goldfish group (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. T-test was used for statistical analysis.

A.5.5. Netting and Restraint Stress Stimulated ACTH, CRF-R1, and NUCB1 mRNA Expression in Goldfish

Netting and restraint stress stimulated the CRF-R1 relative mRNA expression in goldfish whole brain (P value – 0.014) and hypothalamus (P value – 0.022) when compared to controls (**A**). An increase in ACTH mRNA expression in goldfish pituitary (P value – 0.019) (**B**), and NUCB1 mRNA expression in goldfish brain (F value – 30.32; P value – 0.018), hypothalamus (P value – 0.043) and pituitary (P value – 0.023) (**C**) was found in stressed fish compared to controls.

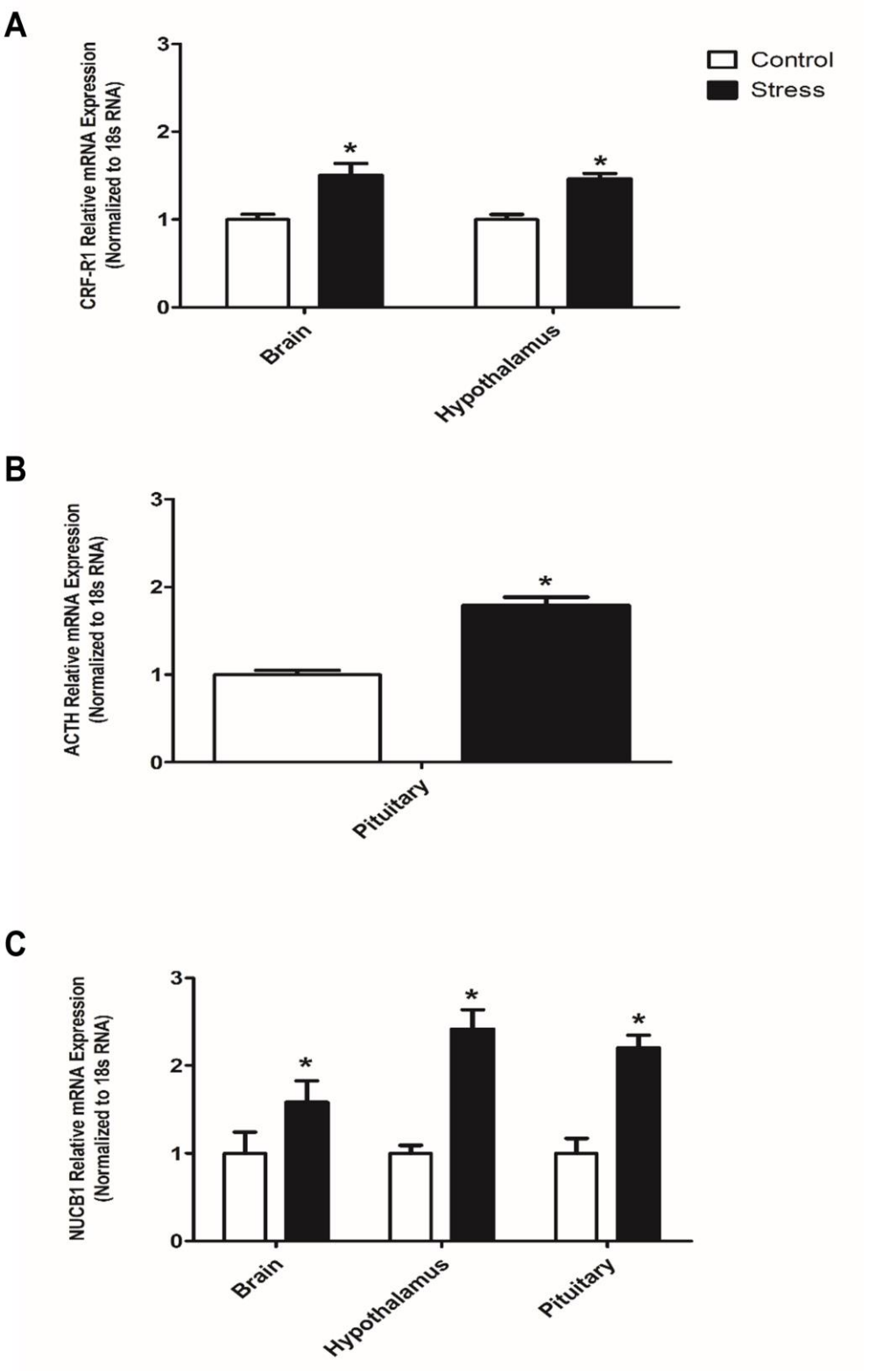

A
CRF-R1 Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
Control
Stress
*
*
Brain
Hypothalamus
B
ACTH Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
*
Pituitary
C
NUCB1 Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
*
*
*
Brain
Hypothalamus
Pituitary

Figure A.5. Netting and restraint stress stimulated ACTH, CRF-R1 and NUCB1 relative mRNA expression in goldfish. Groups include: Control (white bar) and Stressed (black bar) goldfish and the relative mRNA expression of ACTH, CRF-R1 and NUCB1 was normalized to 18s RNA. Asterisks denote significant differences between control (saline group) and stressed goldfish group (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. T-test (**a, b**) and One-way ANOVA and Tukey's multiple comparison test were used for statistical analysis (**c**).

A.5.6. NLP Elevated the Cortisol Levels in Goldfish Post 1-hour and 2-hour of Injection

Goldfish NLP (10 ng/g B.W) elevated the levels of serum cortisol at 1 (P value – 0.02) and 2 hours post-I.P. injection in goldfish when compared to saline injected goldfish (**A**). Administration of goldfish NLP (10 ng/g B.W) elevated the levels of serum cortisol levels post 2-hour of injection in goldfish when compared to controls (P value – 0.044) (**B**).

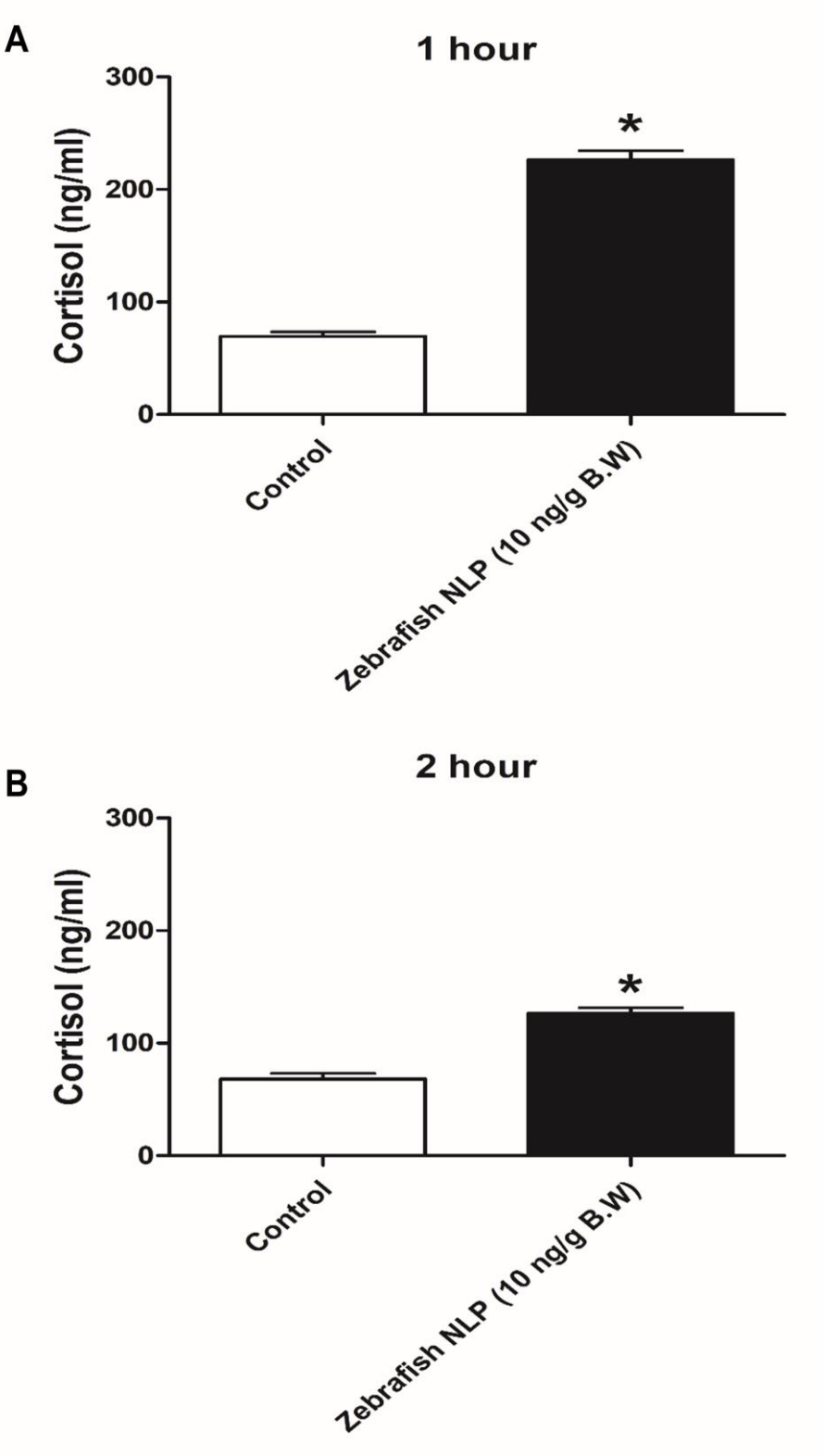
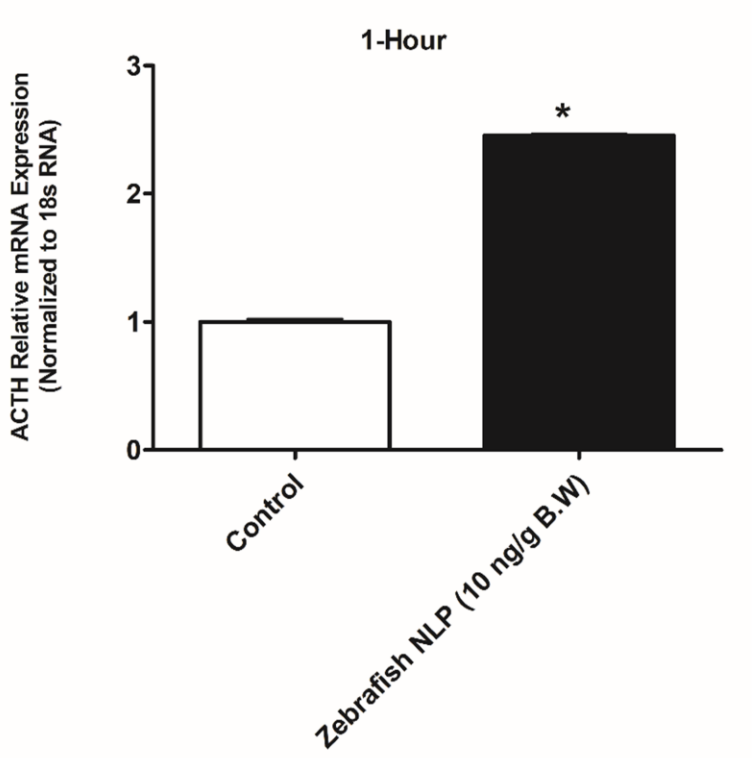

A
1 hour
300
200
100
0
Cortisol (ng/ml)
*
Control
Zebrafish NLP (10 ng/g B.W)
B
2 hour
300
200
100
0
Cortisol (ng/ml)
*
Control
Zebrafish NLP (10 ng/g B.W)

Figure A.6. NLP elevated serum cortisol levels in goldfish post 1-hour and 2-hour of injection in goldfish. Groups include: Control (whit bar) and Dose of zebrafish NLP (10 ng/g B.W) (Black bar) and the cortisol levels were measured in ng/ml at 1 hour (**a**) and 2 hour (**b**) post injection. Asterisks denote significant differences between control (saline group) and NLP-injected goldfish group (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. T-test was used for statistical analysis.

A.5.7. NLP Upregulated ACTH mRNA Expression in Goldfish Pituitary Post 1-hour Injection

Administration of zebrafish NLP (10 ng/g B.W) upregulated ACTH relative mRNA expression in goldfish pituitary post 1-hour injection (P value – 0.0245) when compared to controls (**A**). However, zebrafish NLP (10 ng/g B.W) had no effects on ACTH mRNA expression in goldfish pituitary post 2-hour injection (P value – 0.76) when compared to controls (**B**).

A


1-Hour
ACTH Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
*
Control
Zebrafish NLP (10 ng/g B.W)

B

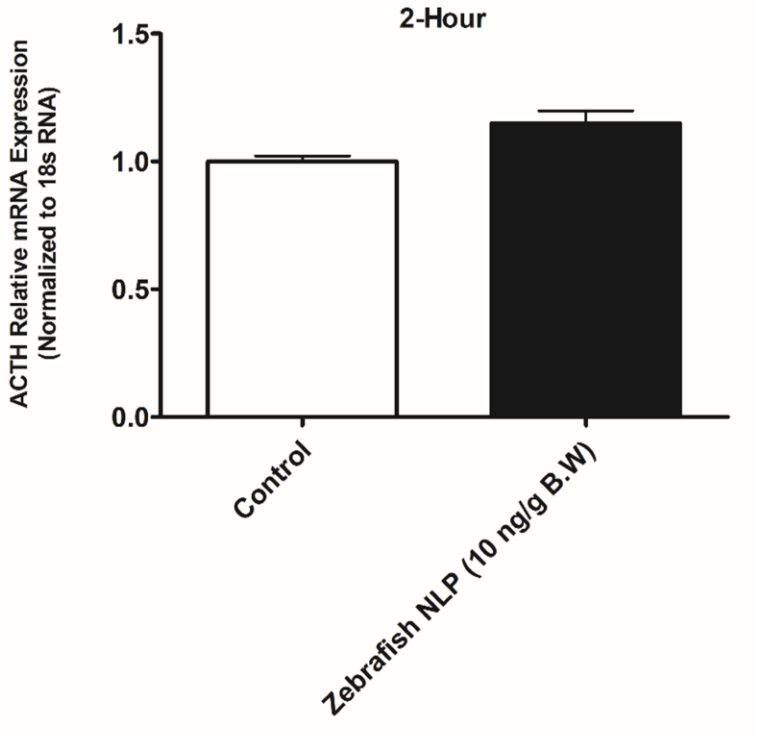

2-Hour
ACTH Relative mRNA Expression
(Normalized to 18s RNA)
1.5
1.0
0.5
0.0
Control
Zebrafish NLP (10 ng/g B.W)

Figure A.7. NLP Administration Upregulated ACTH mRNA in Goldfish Pituitary post 1-hour injection. Groups include: Control (whit bar) and Dose of zebrafish NLP (10 ng/g B.W) (Black bar) and the relative mRNA expression of stress genes were measured at 1 hour (**a**) and 2 hour (**b**) post injection. Data normalized to 18s RNA and represented as mean + SEM. Asterisks denote significant differences between control (saline group) and NLP-injected goldfish group (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. T-test was used for statistical analysis.

A.5.8. NLP Upregulated CRF-R1 mRNA Expression in Whole Brain and Hypothalamus of Goldfish Post 1-hour Injection

Administration of zebrafish NLP (10 ng/g B.W) upregulated CRF-R1 mRNA expression in the whole brain (P value – 0.032) and hypothalamus (P value – 0.0254) of goldfish post 1-hour injection, when compared to controls (**A**). However, zebrafish NLP administration (10 ng/g B.W) did not elicit any effect on CRF-R1 relative mRNA expression in whole brain (P value – 0.65) and hypothalamus (P value – 0.13) of goldfish when compared to controls post 2-hour injection (**B**).

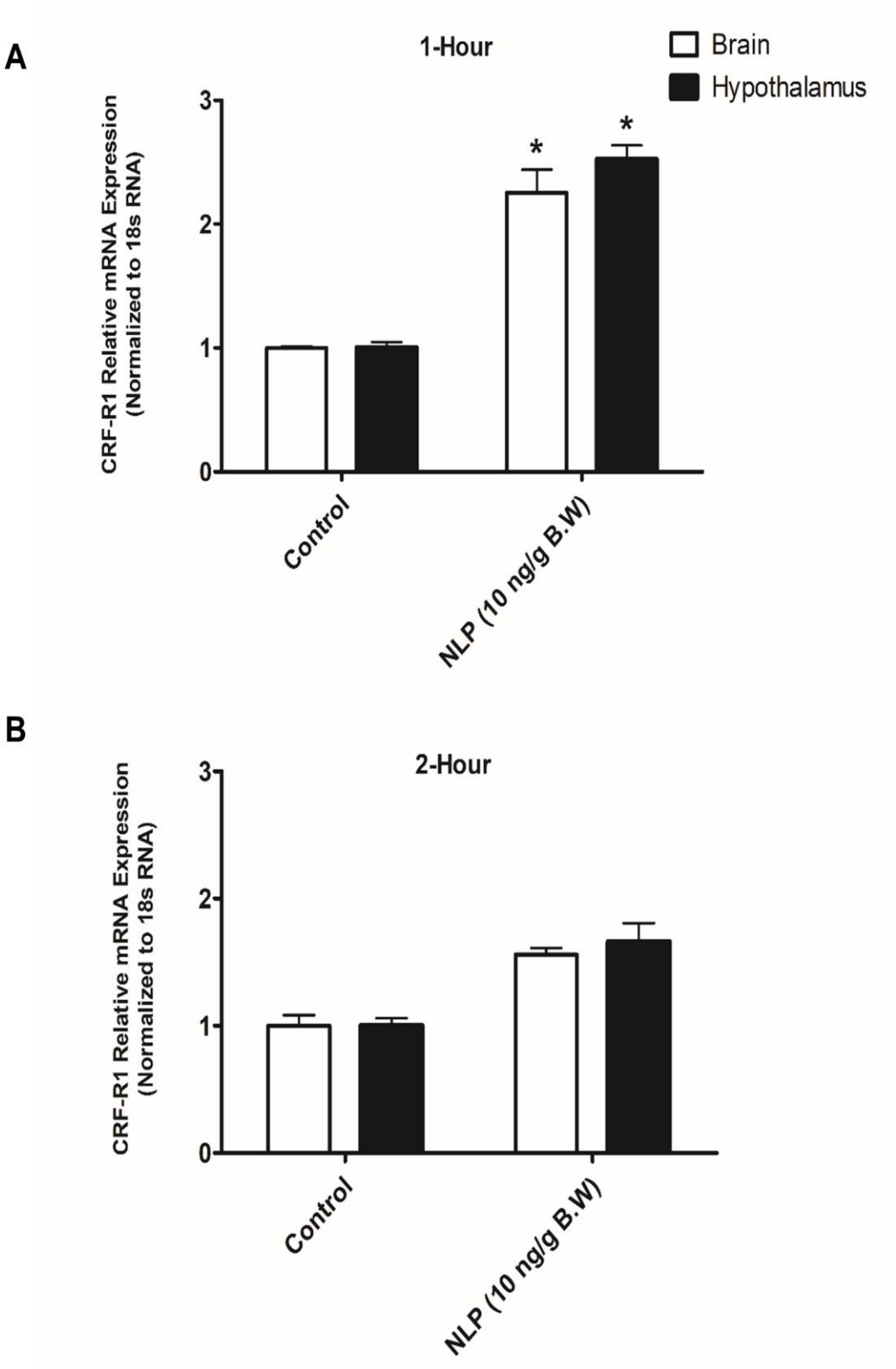

A
1-Hour
Brain
Hypothalamus
CRF-R1 Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
*
*
Control
NLP (10 ng/g B.W)
B
2-Hour
CRF-R1 Relative mRNA Expression
(Normalized to 18s RNA)
3
2
1
0
Control
NLP (10 ng/g B.W)

Figure A.8. NLP Administration Upregulated CRF-R1 mRNA in Goldfish Pituitary post 1-hour injection. Groups include: Control and Dose of zebrafish NLP (10 ng/g B.W) and the relative mRNA expression of stress genes were measured in brain (white bar) and hypothalamus (black bar) at 1 hour (**a**) and 2 hour (**b**) post injection. Data normalized to 18s RNA and were represented as mean + SEM. Asterisks denote significant differences between control (saline group) and NLP-injected goldfish group (* $p<0.05$, n = 6 fish/group). Data are represented as mean + SEM. T-test was used for statistical analysis.

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